Promoters:

Prof. dr. ir. Peter Bossier
Department of Animal Production, Ghent University, Belgium

dr. ir. Peter De Schryver
Department of Animal Production, Ghent University, Belgium

Dean : Prof. dr. ir. Guido Van Huylenbroeck
Rector : Prof. dr. Anne De Paepe

APPLICATION OF POLY-β-HYDROXYBUTYRATE IN GROWTH AND HEALTH PROMOTION OF NILE TILAPIA *Oreochromis niloticus* CULTURE

Magdalena Lenny Situmorang

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Members of the examination and reading committee (*):

Prof. dr. ir. Peter Bossier
Department of Animal Production
Ghent University, Belgium

dr. ir. Peter De Schryver
Department of Animal Production
Ghent University, Belgium

Em. Prof. dr. Patrick Sorgeloos
Department of Animal Production
Ghent University, Belgium

dr. Massimo Marzorati*
Department of Biochemical and Microbial Technology
Ghent University, Belgium

Prof. dr. Marleen De Troch*
Department of Biology
Ghent University, Belgium

Prof. dr. Johan A. J. Verreth*
Aquaculture and Fisheries Department
Wageningen University, the Netherlands

Prof. dr. ir. Guy Smagghe (Chairman)
Department of Crop Protection
Ghent University, Belgium

I dedicate this work to

My parents, Djagomal R. M. Situmorang and Tiur S. Silitonga

My husband, Prawira Partogi Hasian Hutapea

My parents in law, Rustam Hutapea and Lamtiur R. Simatupang

My sisters and brothers, Maria, Ika, Riris, David, Benny

All family members

For their endless love, faith, patience, and encouragement

Notation Index

ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
AOAC	Association of official analytical chemists
aPHB	Amorphous poly-β-hydroxybutyrate
AWCD	Average well colour development
BW	Body weight
CFU	Colony forming unit
CLPP	Community-level physiological profiling
CP	Component principle
cPHB	crystalline poly-β-hydroxybutyrate
DAC	Days after challenge
DAH	Days after hatching
DHA	Docosahexaenoic acid
DMSO	Dimethyl sulfoxide
dpf	Days post fertilization
dpv	Days post vaccination
DSU	Diversity of substrate utilization
DW	Dry weight
EPA	Eicosapentaenoic acid
EPA	Environmental Protection Agency

ESC	Enteric septicaemia
FA	Fatty acid
FAME	Fatty acid methyl ester
FAO	Food and Agriculture Organization
FCR	Food conversion ratio
GC	Gas chromatography
GI	Gastrointestinal
HPLC	High performance liquid chromatography
HUFA	Highly unsaturated fatty acid
MC	Microbial community
NL	Neutral lipid
OD	Optical density
PCA	Principal Component Analysis
PHB	Poly-β-hydroxybutyrate
PL	Polar lipid
PUFA	Polyunsaturated fatty acid
RPS	Relative percentage of survival
SCFA	Short chain fatty acid
SD	Standard Deviation
SGR	Specific growth rate
SIA	Stable isotope analysis
β-HB	β-hydroxybutyrate

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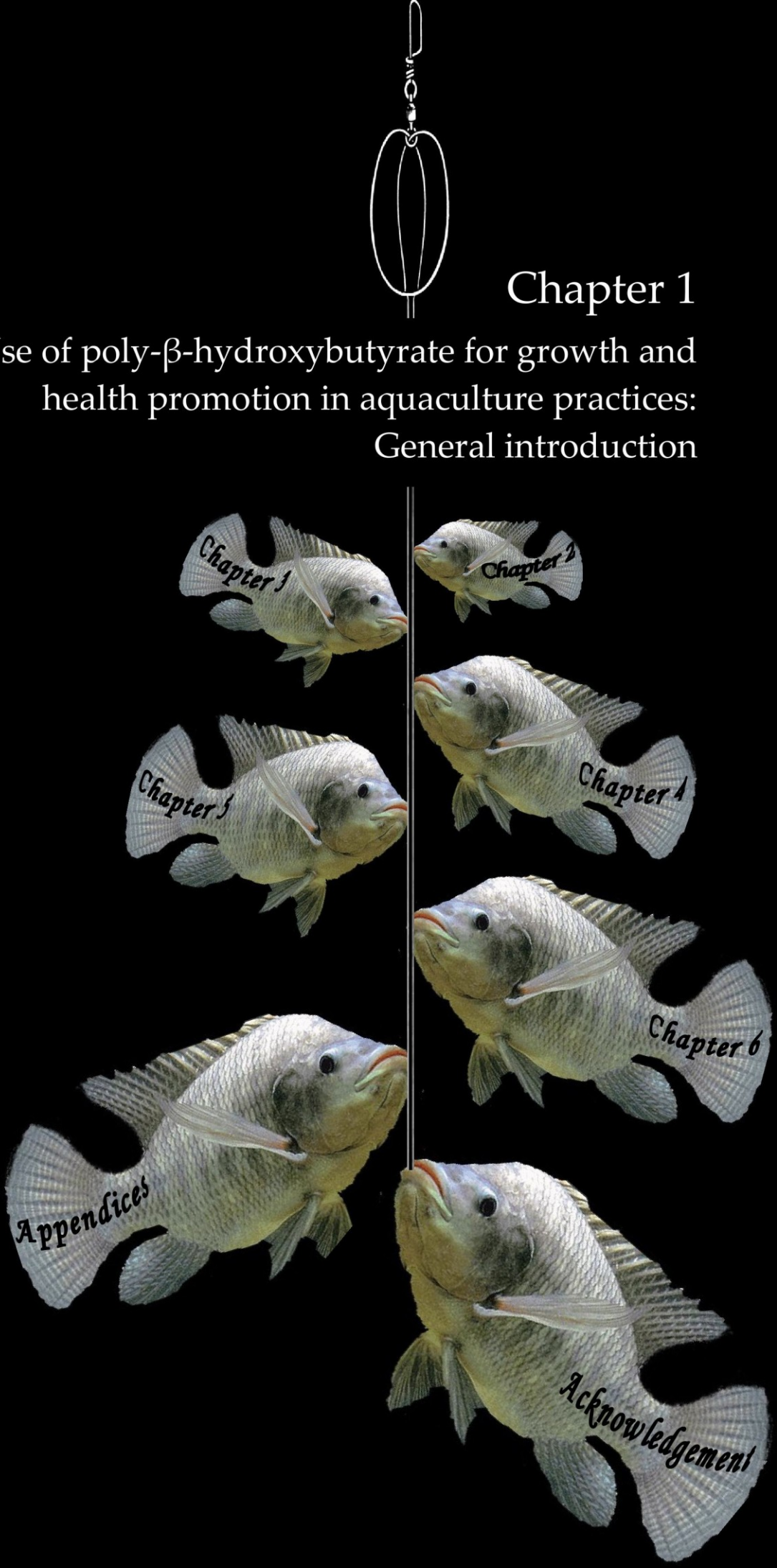


Chapter 1

Use of poly-β-hydroxybutyrate for growth and health promotion in aquaculture practices: General introduction

Status of the world's aquaculture production

It is anticipated that the global human population will rise to 9 billion by 2050 (United Nations, 2011). Beveridge et al. (2013) emphasized that satisfying the food and nutrition needs of the growing population will require a profound change in what and how much is eaten, and where and how food is produced. A failure to do so could profoundly affect the biosphere through biodiversity loss, changes in ecosystem services supply and exacerbated global warming and as such compromise the Earth as a life-support system (Battisti and Naylor, 2009; Foley et al., 2011; Phalan et al., 2011). In the last five decades, global fish production has grown steadily with increasing food fish supply at an average annual rate of 3.2 %, outpacing world population growth at 1.6 % (FAO, 2014). The world *per capita* apparent fish consumption increased from an average of 9.9 kg in the 1960s to 19.2 kg in 2012 (FAO, 2014).

The global wild fish catches have for some time been at or near the limits of what aquatic ecosystems can be expected to provide naturally. Meeting the world's demand for fisheries products has thus depended on the growth of the aquaculture industry, being one of the fastest-growing food producing sectors in the world. In 2012 the aquaculture industry set another all-time high in production as it contributed up to 42 % of the total global fisheries production (Figure 1.1). This share is projected to rise to 47 % of the total global fisheries production in 2022, then up to 62 % by 2030, as catches from wild capture fisheries are levelling off while the demand will substantially increase. In terms of fish destined for human consumption, aquaculture should surpass 50 % of the total fish production by 2015 and reach 53 % by 2022. Developing countries will continue to account for about 67 % of world exports of fish for human consumption, with Asian countries accounting for 54 % of the total (FAO, 2014).

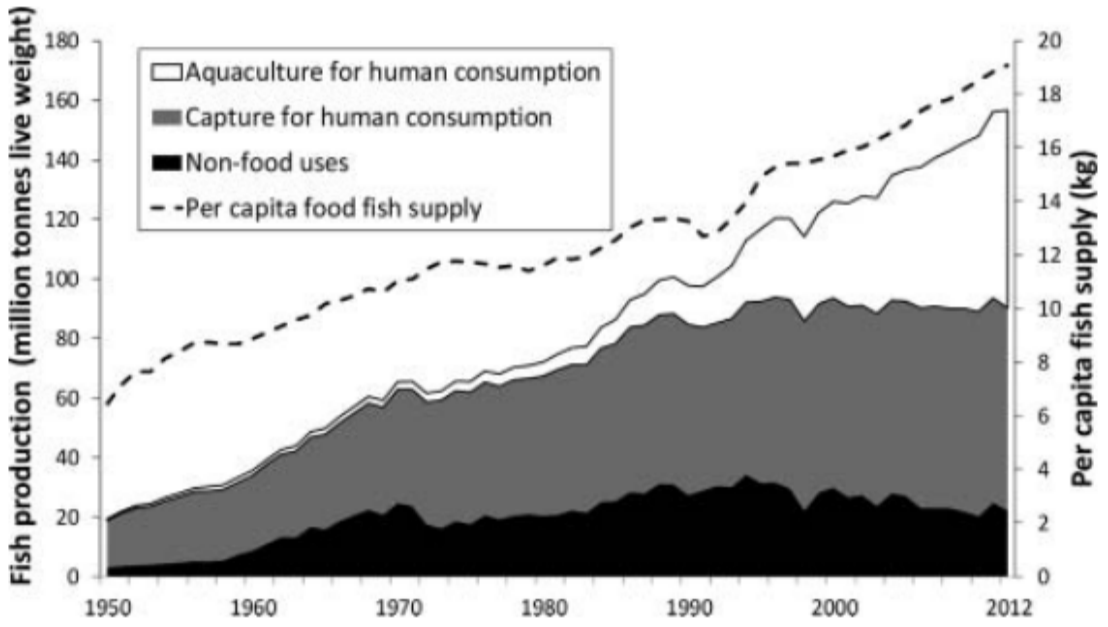


Figure 1.1. Total landings from capture fisheries and aquaculture destined for food and non-food uses and the trends of per capita supply (reproduced from Tacon and Metian, 2015; data from FAO, 2014). *Note*: Production includes, fish, crustaceans, and molluscs, but excludes aquatic plants.

Importance and potential of tilapia species

It is estimated that more than 600 species of aquatic animals are cultured worldwide in a large variety of farming systems, facilities and culture conditions. Aquaculture has allowed the increased consumption of aquatic products by inducing a shift from products being primarily wild-caught to being primarily aquaculture-produced. This has resulted in a raised availability of products such as for shrimp, salmon, bivalves, catfish and tilapia, and a strong increase in their commercialization, thus a decrease in their prices (Beveridge et al., 2013; FAO, 2014). At the global level, looking across fish species, the fastest growth in supply has been observed for tilapia, together with pangasius and Atlantic salmon (Fig. 1.2).

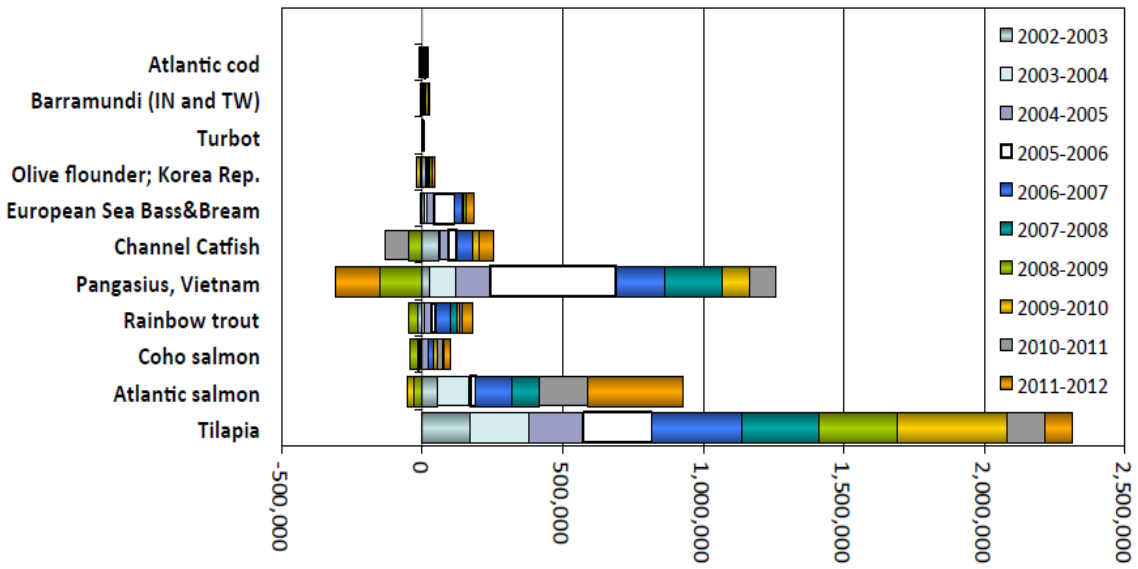


Figure 1.2. Cumulative change in production (metric tonnes) of several major aquaculture fish species from 2002 to 2012 (Nystoyl and Tveteras, 2012).

FAO has recorded farmed tilapia production statistics for 135 countries and territories on all continents (FAO, 2014). Tilapia production is expanding in Asia, South America and Africa (Fig. 1.3), with new supply targeting domestic and regional consumers rather than international markets (FAO, 2014).

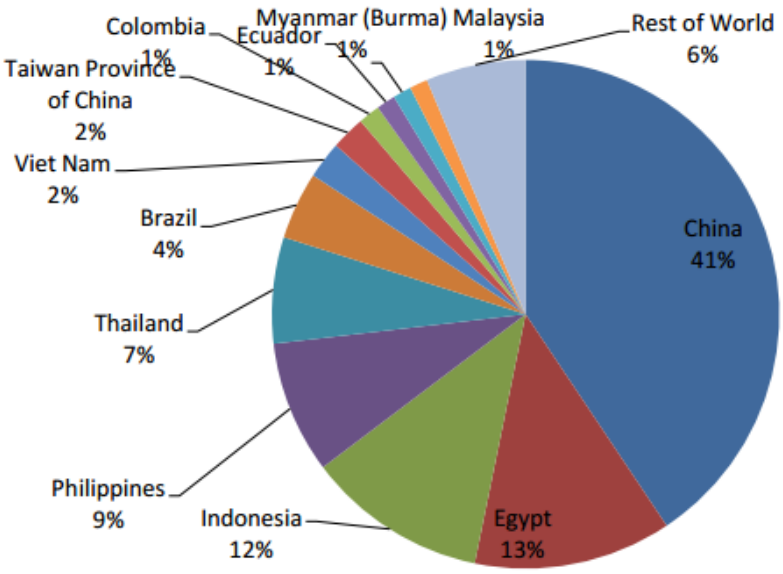


Figure 1.3. Contribution to the total farmed production of 4.85 million tonnes of tilapia per country in 2014 (FAO, 2014)

Several factors have contributed to the rapid global growth of tilapias. Tilapias are easily cultured due to their high reproductive capacity and adaptability to a wide range of environmental conditions such as salinity, dissolved oxygen, temperature, pH, and ammonia levels than most cultured freshwater fishes can (Hussain, 2004). It presents rusticity, good growth rate, and adaptability to confinement, producing a tasty white meat (Oliveira Filho et al., 2010). Tilapia is an omnivorous species and occupies a wide variety of habitats like rivers, lakes and irrigation channels. Tilapia and other farmed aquatic species of low trophic level, i.e. species feeding wildly/naturally lower in the aquatic food chain (including most herbivorous cyprinids, and omnivorous catfish species – all freshwater fish species; Fig. 1.4), are more flexible in terms of feed ingredient use as they can rely on food items from different trophic levels to a varying extent and therefore are less dependent upon the dietary use of fishmeal and fish oil (Tacon and Metian, 2015). Fishmeal and fish oil are continued to be used as the major sources of dietary protein and lipid within compound aquafeeds for the higher trophic level fish and crustacean species (Tacon et al., 2011) (Fig. 1.5). It is expected that, although the strong demand for fishmeal and fish oil in aquaculture diets will continue, the production volumes are likely to remain static or decrease (Tacon and Metian, 2008). Also, there is a growing demand for fishmeal for direct human consumption, and fish oil for direct use in human supplements and pharmaceutical medicines. This market is likely to be able to pay more for fishmeal and fish oil, resulting in aquaculture having to reduce its usage due to the scarcity of these commodities and their increasing prices (Tacon et al., 2012). Tilapia feeds generally contain less than 15 % fish meal, with soybean meal or other alternate protein sources providing the bulk of dietary protein (Luquet, 1991). Thus, increased production of tilapia is also partly due to lower feed prices as compared with those for marine, mostly carnivorous, fish species (Tacon et al., 2009; Tacon et al., 2011).

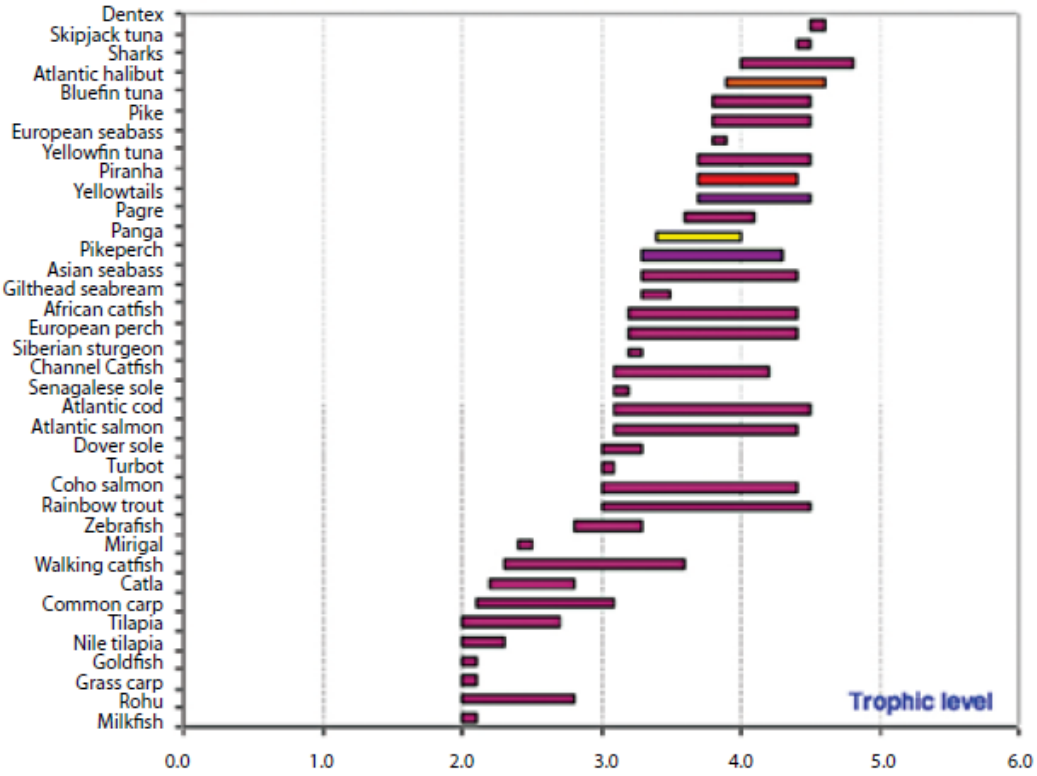


Figure 1.4. Trophic level range for a selection of fish species, including several major aquaculture fish species (Kaushik and Troell, 2010).

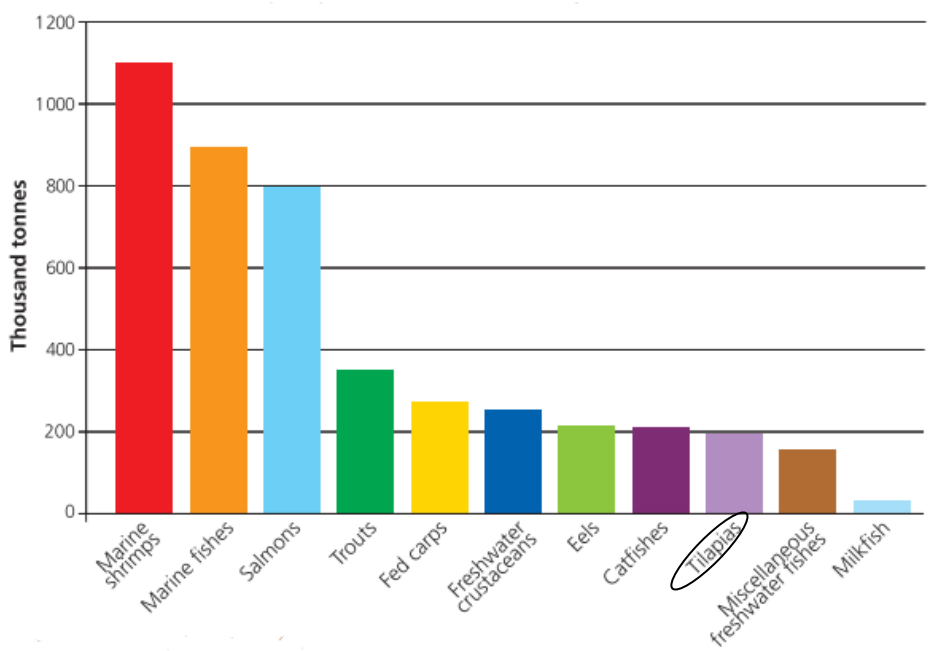


Figure 1.5. Total global consumption of fishmeal and fish oil by major aquaculture species group in 2008 (Tacon et al., 2011)

When raised in optimal conditions, it takes 6-12 months to obtain market-sized tilapia. Out of a total of about 70 tilapia species, nine are used in aquaculture worldwide (FAO, 2002). Nile tilapia (*Oreochromis niloticus*) comprises 83 % of the global production of tilapia and is responsible for the huge expansion of tilapia in recent decades (Table 1.1). The large size at first maturity, which affects the harvest time and thus the market size, and rapid growth rate justify the predominance of the Nile tilapia in their global production (Gupta and Acosta, 2004).

Table 1.1. Total global production of tilapias in 2010 (Tacon et al., 2011)

Species	Production (tonnes)	Percent	Total value (US$)	Value (US$ per kg)
Tilapias (Family Cichlidae)	2,797,819		4,021,164	1.44
Nile tilapia (*Oreochromis niloticus*)	2,334,432	83.4	3,208,561	1.37
Tilapias nei (*Oreochromis* spp.)	419,982	15.0	766,946	1.83
Java tilapia (*O. mossambicus*)	38,140	1.4	32,476	0.85
Blue tilapia (*O. aureus*)	2,687	0.1	5,798	2.16
Three spotted tilapia (*O. andersonii*)	1,996	0.1	5,749	2.88
Longfin tilapia (*O. macrochir*)	187	< 0.1	538.6	2.88
Redbreast tilapia (*Tilapia rendalli*)	160	< 0.1	352	2.20
Redbelly tilapia (*T. zillii*)	130	< 0.1	325	2.50
Sabaki tilapia (*O. spilurus*)	105	< 0.1	420	4.00

Nutrition in tilapia culture

In general, fish meat is an excellent source of high quality protein, essential fatty acids - especially long-chain polyunsaturated fatty acids (PUFAs) - and micronutrients (Kawarazuka and Béné, 2011). The United States Department of Agriculture reported that raw tilapia fillet contains about 20 % protein and 1.7 % fat (USDA, 1987). Fish fatty acids composition, especially essential fatty acids, is also important for human health. Essential fatty acids are unsaturated fatty acids (FAs) that are essential to human health but cannot be synthesized *de novo* in the

body, and thus must be supplied in a ready-made form within the diet (Tacon, 1990). Human consumption of the long chain (n-3) FAs eicosapentaenoic acid (20:5n-3; EPA) and docosahexaenoic acid (22:6n-3; DHA) has been shown to have numerous health benefits, including reduced incidence of heart disease (Hu et al., 2003), improved clinical outcomes in inflammatory diseases such as rheumatoid arthritis (Calder, 2006), and improved outcomes in depression (Freeman et al., 2006). Fish is considered as the major source of (n-3) FAs in the human diet. Although tilapia has a much lower level of the long-chain (n-3) fatty acids as compared to fatty fish such as salmon, they can be considered a good source of these fatty acids relative to beef and chicken. It is well established that many freshwater fish can readily convert linolenic acid (18:3n-3; LLA) to EPA and DHA using metabolic pathways similar to those occurring in higher vertebrates (Anderson and Arthington, 1992). Muinde et al. (2013) reported that Nile tilapia have the ability to bioconvert stearic acid, oleic acid, and other FAs which belong to group C:18 FAs, to highly unsaturated FAs; hence, Nile tilapia is classified as an excellent source of (n-3) PUFAs and being suitable for production of (n-3) supplements.

Intensification of fish production methods increases reliance on nutritionally complete feeds (Tacon and Metian, 2015). Hence, relevant qualitative information on the nutritional requirement of tilapia is critical to adequately supply the required nutrients in the formulated diet for an optimum fish performance. In general, early juvenile tilapia (up to 10 g) require a diet high in protein, lipids, vitamins and minerals and lower in carbohydrates. Sub-adult tilapia (10-25 g) require more energy from lipids and carbohydrates for metabolism and a lower proportion of protein for growth while adult tilapia (> 25 g) require even less dietary protein for growth and can utilize even higher levels of carbohydrates as a source of energy (Tacon and Metian, 2008).

Proteins

Dietary proteins are used continuously by fish for maintenance, growth, and reproduction functions. When fed in excess, protein may be used as energy. However, the latter function is not desirable because of the cost of proteins. Several factors including fish size or age, dietary protein quality, energy content of the diet, water quality and culture conditions have been reported to affect protein requirements of tilapia. Many studies have indicated that the protein requirement for maximum performance of tilapia during larval stages is relatively high (35 % to more than 50 %), and decreases with increasing fish size (El-Sayed and Teshima, 1992; Jauncey and Ross, 1982; Siddiqui et al., 1988; Winfree and Stickney, 1981). For tilapia juveniles, the protein requirement ranges from 30–40 %, while adults require 20–30 % dietary protein for optimum performance (El-Sayed and Teshima, 1991). Tilapia broodstock require 35–45 % dietary protein for optimum reproduction, spawning efficiency, and larval growth and survival (El-Sayed et al., 2003; Gunasekera et al., 1996a; Gunasekera et al., 1996b; Siddiqui et al., 1998).

The profile of dietary protein is important when formulating diets for tilapia. As for other tropical fish, tilapia require essential amino acids that need to be supplied by the diet (NRC, 1983) (Table 1.2). Essential amino acid requirements can be met by the use of a balance of both plant and animal proteins, and if necessary, by the inclusion of synthetic amino acids in the complete feed. Several feeding studies reported the benefit of dietary supplementation with free amino acids on feeding intake and growth of tilapia (El-Saidy and Gaber, 2002; Yacoob et al., 2001).

Table 1.2. Essential amino acid requirements of *O. mossambicus* and *O. niloticus* as % of dietary protein and of total diet (in parenthesis).

Amino Acid	Requirement			
	O. mossambicus[1]	*O. mossambicus*[2]	*O. niloticus*[3]	*O. niloticus*[4]
Lysine	4.05 (1.62)	3.78 (1.51)	5.12 (1.43)	*na*
Arginine	3.80 (1.52)	2.82 (1.13)	4.20 (1.18)	4.1
Histidine	*na*	1.05 (0.42)	1.72 (0.48)	1.5
Threonine	*na*	2.93 (1.17)	3.75 (1.05)	3.3
Valine	*na*	2.20 (0.88)	2.80 (0.78)	3.0
Leucine	*na*	3.40 (1.35)	3.39 (0.95)	4.3
Isoleucine	*na*	2.01 (0.80)	3.11 (0.87)	2.6
Methionine	1.33 (0.53)	0.99 (0.40)	2.68 (0.75)	1.3
Cystine	*na*	*na*	0.53	2.1
Phenylalanine	*na*	2.50 (1.00)	3.75 (1.05)	3.2
Tyrosine	*na*	*na*	1.79	1.6
Tryptophan	*na*	0.43 (0.17)	1.00 (0.28)	0.6

'na' indicates data is not available.
[1]Jackson and Capper (1982); [2]Jauncey et al. (1983), [3]Santiago and Lovell (1988); [4]Fagbenro (2000).

Carbohydrates

Carbohydrates represent a broad group of substances which include sugars, starches, gums and celluloses. Carbohydrates make up three-fourths of the biomass of plants but are present only in small quantities in the animal body as glycogen, sugars and their derivatives. Carbohydrates present an inexpensive energy source that would "spare" the catabolism of other components such as non-essential amino acids and fatty acids for energetic purposes (Dupree and Hunter, 1984). Warm water fish are known for their ability to use higher amounts of dietary carbohydrate than cold water and marine species (NRC, 1983). The utilization of carbohydrate by tilapia appears to differ depending on the complexity of these carbohydrates. Starch or dextrin (partially hydrolysed starch) is used more efficiently by tilapia than sugars such as glucose or sucrose (Edwin and Meng, 1996). Although tilapias use carbohydrate effectively, there is no dietary requirement for carbohydrate.

Lipids

Dietary lipids provide a major source of energy, facilitate the absorption of fat soluble vitamins, play an important role in membrane structure and function, serve as precursors for steroid hormones and prostaglandins, and serve as metabolizable sources of essential FAs. Lipids are also considered important in the flavour and textural properties of the feed consumed by fish (NRC, 1983). The type and amount of lipid used in tilapia diets are based on essential FAs requirements, economic constraints of feed manufacture and quality of fish flesh desired (Eyo, 2002). Jauncey (2000) suggested that to maximize protein utilization, dietary fat concentration should be between 8 and 12 % for tilapia with size <25 g, and between 6 to 8 % for larger fish (>25 g). As with most fish, tilapia has the requirement for (n-6) FAs, and to a lesser extent, a requirement for (n-3) FAs. Dietary lipids should supply (n-6) FAs at 0.5 – 1 % dry feed weight (Teshima et al., 1982). Farmed fish have become very important in providing health-promoting (n-3) polyunsaturated fatty acids (PUFA) for the consumers. Generally, wild tilapias have more (n-3) FAs, higher (n-3)/(n-6) PUFA ratios, with lower proportions of 18:2n-6 than farmed tilapias (Karapanagiotidis et al., 2006), but diet adjustments can alter the body composition of the domesticated variety (Young, 2009). It is reported that the amount and composition of external feed used, which indicate the intensity of the fish production methods, affects fish lipid content and composition (Karapanagiotidis et al., 2006; Weaver et al., 2008).

Feed additives as feeding and growth enhancer

Significant price increases have occurred during the past years for major aquafeed ingredients, including fishmeal and fish oils (Tacon and Metian, 2008). In the aquaculture sector, the combined efforts of industry and academic institutes resulted in the development of a wide range of additives to improve nutrient utilization and reduce feed formulation costs. Various types of feed additives, including exogenous enzymes and feeding stimulants, are used to enhance the

digestibility and/or utilization efficiency of nutrients (Drew et al., 2005; Farhangi and Carter, 2007; Lin et al., 2007). The use of enzyme products as animal feed supplements is of considerable interest to feed manufacturers and animal producers as a means to improve animal performance and reduce feed costs (Felix and Selvaraj, 2004; Feord, 1996; Forster et al., 1999; Lin et al., 2007). Goda et al. (2012) reported that the use of a mixture of exogenous digestive enzymes enhanced the growth of Nile tilapia fingerling with up to 30 %, increased the nutrient utilization, i.e. lower feed conversion ratio (FCR) and higher protein efficiency ratio (PER), and improved some hematological parameters including increase in total plasma protein (TPP) and total plasma globulin (TPG) level. The higher TPP and TPG levels of 2.8-3.6 g dL^{-1} and 1.5-2.2 g dL^{-1}, respectively, were observed in fish fed different diets (supplemented with mixture of pepsin, papain and α-amylase at different concentrations), compared to 2.7 g dL^{-1} and 1.4 g dL^{-1}, respectively, in fish fed the control diet. Various studies suggested that the addition of phytase into tilapia diets can improve growth performances by protecting amino acids from degrading, by decreasing the leaching of water soluble components and by improving the digestibility and utilization of dietary protein and phosphorous (Heindl et al., 2004; Liebert and Portz, 2004; Phromkunthong et al., 2004; Riche et al., 2001). Other types of feed additives including immunostimulants and pro-/prebiotics have also been studied and used to stimulate fish growth and digestion, along with their application in disease management (Balca´zar et al., 2006; Bricknell and Dalmo, 2005; Kesarcodi-Watson et al., 2008; Nayak, 2010; Sakai, 1999; Tinh et al., 2008; Verschuere et al., 2000; Villamil and Novoa, 2009).

Diseases in tilapia culture

As aquaculture becomes more intensive, new diseases are likely to emerge, and old diseases will appear in new locations. In intensive aquaculture systems, environmental and microbial conditions deteriorate rapidly causing significant stress to the aquatic animals (Conte, 2004; Subasinghe, 2005). Infectious diseases are by far the main reason for mortality of farmed fishes. An outbreak often wipes out

an entire stock and it requires costly decontamination of the associated facilities and equipment to restore the situation (Pillay and Kutty, 2005). Diseases can be considered a significant limiting factor to aquaculture production. Several diseases have been reported to affect tilapia culture during different stages of the farming cycle (Fig. 1.6).

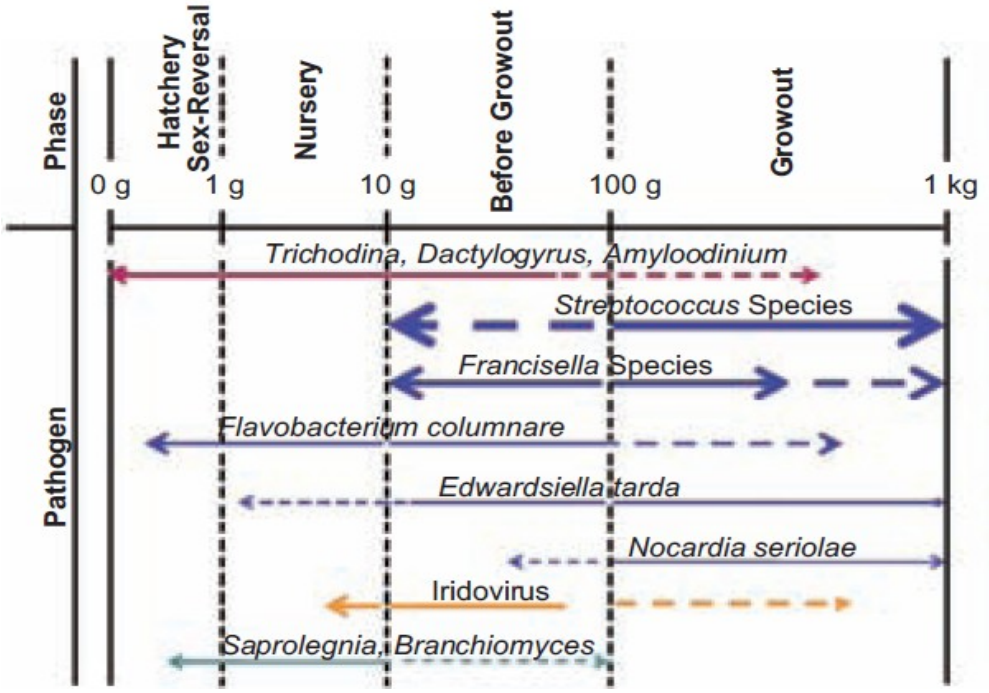


Figure 1.6. Main disease causative agents affecting tilapia during the farming cycle. Importance of the disease is roughly in proportion to the size of the arrow bars (Komar, 2008).

Bacterial diseases

Tilapia have long been considered a species that is ideal for use in aquaculture due to its hardy nature, fast growth, tolerance of suboptimal water quality and disease resistance (Little et al., 2008). However, tilapia also suffer from diseases that are not always new problems, but old problems that are re-emerging due to intensive production practices. Bacterial diseases are the main cause of mortality in tilapia culture, often resulting from an infection with a secondary or opportunistic agent combined with an infection by parasites. Some bacterial diseases have a seasonal

pattern, while others are not necessarily related to climatic factors or may occur throughout the whole cycle (Komar, 2008; Noga, 2011; Shoemaker and Klesius, 1997). Streptococcosis and francisellosis are examples of seasonal bacterial diseases, primarily related to water temperature. Streptococcosis, also known as infectious meningoencephalitis caused by Gram-positive *Streptococcus agalactiae*, and to a lesser extent *S. iniae*, has been responsible for significant losses in intensive tilapia culture. As a typical feature the disease can occur during a period in which the water temperature is above 26 °C (Perera et al., 1994; Shoemaker and Klesius, 1997). Affected fish are described as having erratic swimming patterns, pericarditis, and chronic inflammatory reaction, hemorrhagic meningitis, polyserositis, or fibrosis. Francisellosis, also known as visceral granulomatosis, is caused by *Francisella noatunensis* and outbreaks particularly occur in periods with water temperature below 24 °C (Jeffery et al., 2010).

Columnaris disease is another important cause of mortality in tilapia rearing and is responsible for large economic losses worldwide. It is caused by *Flavobacterium columnare*, resulting in skin lesions, fin erosion and gill necrosis, with a high degree of mortality (Decostere et al., 1998). *F. columnare* infection is highly influenced by fish culture management (Declercq et al., 2013). Abrupt variations in water temperature are likely to induce and accelerate the progression of this disease. Poor water quality, inadequate diet, handling and overcrowding are also stress factors likely to induce an outbreak (Pilarski et al., 2008; Sebastião et al., 2010). Septicemia diseases can also strike tilapias, due to a variety of pathogens including *Aeromonas hydrophila* (Gado, 1998; Plumb and Hanson, 2011), *Pseudomonas* sp. (El-Attar and Moustafa, 1996; Miyazaki et al., 1984; Soto-Rodriguez et al., 2013) and *Edwardsiella* sp., namely *E. tarda* (Clavijo et al., 2002; Iregui et al., 2012; Miyashita, 1984) and *E. ictaluri* (Situmorang et al., 2014; Soto et al., 2012; Soto et al., 2013). These are facultative pathogens (i.e. pathogenicity appears to be related to the stress of a weakened hosts) that can be controlled by good aquaculture management (Danner and Merrill, 2006; Komar, 2008; Plumb and Hanson, 2011).

Fungal diseases

Fungal diseases in tilapia, particularly saprolegniasis, can be found throughout all stages of tilapia production (Aly and El-Ashram, 2000; El-Sayed, 2006; Noor et al., 2010; Zaki et al., 2008). Saprolegnia is dermatotropic as it affects the skin and can penetrate to the dermal *stratum compactum*. Another fungal disease that affects tilapia is aspergillomycosis, which is primarily seen in animals that have been fed pellets that are already contaminated by the fungus and can result in high mortality (Olufemi et al., 1983; Olufemi and Roberts, 1986; Refai et al., 2010). Fungal diseases (mycosis) used to be considered a primary disease, but currently can be considered secondary to management practices, particularly during transfer or handling of the fish.

Parasitic diseases

Several external parasites, including protozoan *Trichodina,* metazoan *Gyrodactylus* and dinoflagellate *Amyloodinium* have been identified as major disease pathogens in tilapia culture (Shoemaker et al., 2006). Parasites commonly cause problems in young fish stocked at high numbers in eutrophic waters (Klesius and Rogers, 1995; Shoemaker et al., 2006). Several studies suggested that parasites are able to vector viral and bacterial diseases of fish, increasing the transmission efficiency of pathogens by creating portals of entry and/or by having the ability to transfer pathogens directly from fish to fish (Bandilla et al., 2006; Cusack and Cone, 1986; Evans et al., 2007; Kanno et al., 1990; Pylkkö et al., 2006). Other studies suggest different mechanisms that increase host susceptibility; for example, parasitism has been shown to result in increased stress responses believed to be linked to decreased disease resistance (Bowers et al., 2000; Tully and Nolan, 2001).

Viral diseases

Iridovirus is the only documented viral disease in tilapia. A Bohle iridovirus case in tilapia was reported in Australia (Ariel and Owens, 1997). A disease outbreak due to the iridovirus has also been found in Canada in tilapia fingerlings imported from

the USA (McGrogan et al., 1998). Iridovirus infection may cause immuno-suppression, therefore facilitating other diseases caused by other pathogens.

Management of diseases in tilapia culture

Hygienic prevention strategies

The best way to control infectious diseases is to avoid their introduction. Several ways to avoid the introduction of fish pathogens are (1) using fishes free from pathogens (i.e. specific pathogen free or SPF fish), (2) using foods free of pathogens (live foods can be vectors for fish pathogens), (3) application of prophylactic treatments with therapeutic agents via static baths, parental drug administration, or orally via medicated feed to remove external parasites, treat a diagnosed disease, or reduce shedding of pathogenic agents, and (4) filtration and disinfection of incoming water to the main system by use of for example ultraviolet light sterilization or ozonation (Kent et al., 2009).

Disinfection procedures of culture water and equipment have been widely applied to prevent the introduction and spread of infectious disease (Danner and Merrill, 2006). Commonly used chemical disinfectants in aquaculture are inorganic chlorine products (bleach, as sodium hypochlorite or calcium hypochlorite), buffered potassium monopersulphate (e.g. Virkon® Aquatic), surfactants (e.g. quaternary ammonium compounds), biguanides (e.g. chlorhexidine), alcohols (e.g. ethanol, isopropyl alcohol), iodophors (e.g. Betadine®), carboxylic acids (e.g. citric or acetic acids), oxidizing agents (e.g. hydrogen peroxide), phenol derivatives (e.g. Lysol®), and formaldehyde (Danner and Merrill, 2006; Noga, 2011; Somga et al., 2012). Chemical disinfectants are useful against specific disease organisms, although their effectiveness might vary depending on specific doses and/or contact times (Yanong and Erlacher-Reid, 2012).

Typical water treatment systems make use of high efficiency sand filters to clarify the water before treatment with ultraviolet (UV) light or ozone (O_3). The effectiveness and cost of such a system will depend on many factors including the

volume of water to be treated, the targeted disease(s), and the contact time of the treatment (Pietrak et al., 2010). UV irradiation intends to destroy the DNA of microorganisms, causing death or inactivation (Liltved, 2002). The dose of UV irradiation necessary to inactivate different microorganisms depends on the irradiation intensity and exposure time. For example, *Saprolegnia* requires UV doses that are 4 to 10 fold higher than the level needed for bacteria in order to achieve inactivation (Sharrer et al., 2005). UV irradiation has been found to be the cheapest method of sterilizing the water, especially for egg incubation systems (Bhujel, 2014). However, the turbidity caused by organic products is a major problem for efficient penetration of UV irradiation in the water. Another problem associated with this technique is that it does not only target the pathogens but also the beneficial bacteria (Summerfelt, 2003).

Ozonation is another option for disinfecting water by destroying the outer membranes of bacterial and fungal pathogens in aquatic systems (Graslund and Bengtsson, 2001) and can also inactivate viruses. Ozone also enhances water clarity by breaking down dissolved and particulate organics that discolour the water and that could clog the mechanical and biological filters or could provide a food source for undesirable bacteria and other pathogens (Yanong and Erlacher-Reid, 2012). Summerfelt et al. (2009) reported that a daily application of ozone with a dose of 0.34-0.39 mg L^{-1} decreases the total cultivable heterotrophic bacteria to almost zero. However, ozonation is relatively costly and its by-products including bromate and other brominated compounds are considered toxic for fish and humans (Bhujel, 2014; Tango and Gagnon, 2003). Similar with UV irradiation technique, the risk of elimination of beneficial bacteria is a major concern with the ozonation technique (Liltved, 2002; Summerfelt, 2003).

Prevention strategies acting at the host immunity level

Vaccination

The use of antimicrobials may be significantly reduced by the use of vaccines, when possible (Gudding, 2012). These are products that are directly or indirectly produced from pathogen biomass and administered to the animal to elicit a specific immune response for the prevention of a range of mainly bacterial and viral diseases. Vaccinations can be supplied as immersion, oral or by injection preparations and can minimize the effects of disease, which in turn minimizes morbidity and mortality, promotes optimum growth and improves feed conversion (Sommerset et al., 2005). A number of factors are known to influence the ability of vaccines to protect fish. These include environmental factors such as temperature and pollutants; host factors such as age and general health; husbandry factors such as handling, stress, diet and antibiotics; and vaccine-related factors such as dose, nature of antigen, route of administration and presence of adjuvants (Karunasagar and Karunasagar, 1999). In tilapia culture specifically, vaccination has offered protection to bacterial diseases (Table 1.3).

Unlike the successful bacterial vaccines which are now routinely used in aquaculture, only very few anti-viral vaccines are available (mostly for salmonids) (Christie, 1997; Dhar and Allnutt, 2011; Lorenzen et al., 1998; Winton, 1997). Furthermore, although parasites cause significant problems in the aquaculture industry, there are no commercial vaccines for parasitic infections (Karunasagar, 2012). Vaccines may be developed against parasites (Vercruysse et al., 2007) or fungal diseases (Minor et al., 2014), but not in the near future. Development of such vaccines will allow antibiotics and chemotherapeutants to be reserved for emergencies.

Immunostimulants

An immunostimulant is a naturally occurring compound that modulates the immune system by increasing the host's resistance against diseases that in most circumstances are caused by pathogens (Bricknell and Dalmo, 2005). Immunostimulants can be divided into several groups depending on their sources: bacterial, algae-derived, animal-derived, nutritional factors and hormones/cytokines (Sakai, 1999). The enhancement of phagocytic cells activity, lymphocytes, complement activity and antibody production by immunostimulants has been reported in several studies (Aakre et al., 1994; Hardie et al., 1991; Sakai et al., 1993; Siwicki et al., 1996). There are numerous studies on immunostimulants on tilapia culture, and most of them report improved resistance to challenge by various bacterial pathogens, with observed growth modulation in several studies (Table 1.4).

In fish larviculture, delivery of immunostimulants is generally by bath immersion or through fish oral administration. Pulse feeding is commonly practiced. Some concerns have been raised about the use of immunostimulants in larval fish, in which there could be induction of tolerance. Problems also exist with respect to delivery system for fish larvae in flow-through systems and the effect on biological filters in recirculating aquaculture systems. Considering the variability in response in different fish species, the interval of feeding in the pulse feeding strategy is another issue that needs to be resolved (Bricknell and Dalmo, 2005).

Table 1.3. Use of vaccines for health promotion in Nile tilapia culture

Vaccine	Life stage	Administration procedure	Effect	Reference
Formalin killed cells of *Aeromonas hydrophila*	Adult	Intraperitoneal (i.p.) injection at 10^6 CFU $fish^{-1}$	– Relative percentage of survival (RPS) of 62 % after challenge with virulent *A. hydrophila* (i.p. injected at $1.5x10^4$ CFU $fish^{-1}$) on 7 days post vaccination (dpv)	Ruangpan et al. (1986)
Live attenuated *Edwardsiella tarda* SPM31	Fingerling	i.p. injection at 1 mg kg^{-1} body weight	– RPS of 100 % after challenge with virulent *E. tarda* FPC498 (i.p. injected at at 1 mg kg^{-1} body weight)	Igarashi and Iida (2002)
Attenuated *Streptococcus agalactiae*	Adult	i.p. injection at 10^6 CFU $fish^{-1}$	– RPS of 40 % after challenge with virulent *S. agalactiae* (i.p. injected at $1.5x10^4$ CFU $fish^{-1}$ on 90 dpv)	Pasnik et al. (2006)
Attenuated *S. iniae* ISET0901	Adult	i.p. injection at $2x10^7$ CFU $fish^{-1}$	– RPS of 75 % after challenge with virulent *S. iniae* ISET0901 (i.p. injected at $2x10^7$ CFU $fish^{-1}$ on 180 dpv)	Pridgeon and Klesius (2011)
Attenuated *Francisella asiatica*	Adult	i.p. injection at 10^4 CFU $fish^{-1}$	– RPS of 90 % after challenge with virulent *F. asiatica* (i.p. injected at 10^6 CFU $fish^{-1}$)	Soto et al. (2011)
Mixture of extracellular product and sonicated cell of *Pseudomonas fluorescens*	Adult	i.p. injection at $8x10^7$ CFU $fish^{-1}$	– RPS of 60 % after challenge with virulent *P. fluorescens* (i.p. injected at $4x10^8$ CFU $fish^{-1}$ on 42 dpv)	Attia et al. (2012)
Avirulent *F. columnare* genomovar II mutant; developed by rifampicin-resistance strategy	Juvenile	Immersion at $2x10^6$ CFU ml^{-1} for 30 min	– RPS of 87 % after challenge with virulent *F. columnare* ARS-1 (immersion at $5.5x10^6$ CFU mL^{-1} on 28 dpv)	Mohammed et al. (2013)

Treatment strategies acting at the microbial level

Antimicrobials

A number of antimicrobials have been used in the aquaculture industry as curative agents of infectious bacterial diseases and for prophylactic purposes (Kesarcodi-Watson et al., 2008). The use of various antimicrobial agents in Nile tilapia hatcheries and grow-out facilities have been reported, mostly in Asian countries, including the use of antifungals (e.g. formalin, malachite green, trifuralin, methylene blue), antiparasitics (e.g. belzalkonium chloride, copper sulphate, potassium permanganate, sodium chloride) and antibiotics (e.g. amoxicillin, doxycycline, erythromycin, enrofloxacin, sulfaquinoxaline, trimethoprim-sulfadiazine, florfenicol, norfloxacin, oxytetracycline) (Somga et al., 2012; Yuan and Chen, 2012).

Regular use of antibiotics in aquaculture is not a sustainable practice. There are possible adverse effects of the use of antibiotics including significant resistance in target bacteria, resulting in ineffective and imprudent treatments (Defoirdt et al., 2007a; Karunasagar, 2012). Antibiotics are also often used in the wrong way and therefore lead to ineffectiveness, for example when applied orally while infected fish display a reduced appetite. Their routine prophylactic use, particularly in hatcheries, is often uneconomic and unjustifiable with the absence of an accurate diagnosis and the fact that the pathogen was often not the primary cause of the disease process (Yuan and Chen, 2012).

Table 1.4. Use of immunostimulants for health and growth promotion in tilapia culture (part 1)

Antagonist / active compound	Life stage / size	Disease causative agent	Effect	Reference
Algae-derived				
Spirulina *(Arthrospira platensis)*	Fry (~1.9 g)	*Aeromonas hydrophila*	– Increase in growth – Increase in survival with 10 to 70 %	Abdel-Tawwab and Ahmad (2009)
Spirulina platensis	Fingerling (~50 g)	*A. hydrophila*	– Increase in phagocytic activity – Increase in survival with 40 to 70 %	Ragap et al. (2012)
S. platensis	Fingerling (4.0±1.0 g)	*Pseudomonas fluorescens*	– Increase in survival with 12 to 25 % – Increase in growth	Ibrahem et al. (2013)
Jade Spirulina® *(S. platensis)*	Fingerling (50±5 g)	*Vibrio alginolyticus*	– Increase in survival with 25 to 55 %	Abdel-Latif and Khalil (2014)
Fungi/Yeast-derived				
Scleroglucan (from fungus *Sclerotium rolfsii*)	Fingerling (21.8±3.3 g)	*A. hydrophila*	– Increase in survival with 70 %	Wang and Wang (1997)
Scleroglucan (from fungus *Sclerotium rolfsii*)	Fingerling (21.8±3.3 g)	*Edwardsiella tarda*	– Increase in survival with 80 %	Wang and Wang (1997)
Saccharomyces cerevisiae	Fingerling (21.9±0.3 g)	*A. hydrophila*	– Increase in survival with 80 %	Rasha et al. (2010)
β-glucan *from Saccharomyces cerevisiae*	Adult (80-100 g)	*A. hydrophila*	– Increase in survival with 55 %	El-Boshy et al. (2010)
Saccharomyces cerevisiae cell wall	Adult (125.0±1.5 g)	*Streptococcus agalactiae*	– Increase in survival with 28 to 38 %	Salvador et al. (2012)

Table 1.4. Use of immunostimulants for health and growth promotion in tilapia culture (part 2)

Antagonist / active compound	Life stage /size	Disease causative agent	Effect	Reference
Plant-derived				
PS-K (protein-bound polysaccharide) extracted from mushroom *Coriolus versicolor*	Fingerling (30–50 g)	*E. tarda*	– Increase in phagocytic activity – Increase in survival with 30 to 100 %	Park and Jeong (1996)
Indian almond *Terminalia catappa* extract	Egg	Fungus	– Decrease in fungal infection with 8 %	Chitmanat et al. (2005)
Combination of Chinese herbs (*Lonicera japonica* and *Ganoderma lucidum*)	Fingerling (52.5±3.5 g)	*A. hydrophila*	– Increase in survival with 37 %	Yin et al. (2008)
Green tea (*Camellia sinensis*)	Fingerling (1.5–2.0 g)	*A. hydrophila*	– Increase in survival with 25 to 70 % – Increase in growth	Abdel-Tawwab et al. (2010)
Coneflower (*Echinacea purpurea*)	Juvenile (0.8±0.2 g)	*A. hydrophila*	– Increase in survival with 25 to 45 % – Increase in growth	Aly and Mohamed (2010)
Garlic (*Allium sativum*)	Juvenile (0.8±0.2 g)	*A. hydrophila*	– Increase in survival with 15 to 35 % – Increase in growth	Aly and Mohamed (2010)
Cinnamon (*Cinnamomum zeylanicum*)	Fingerling (10–12 g)	*A. hydrophila*	– Increase in survival with 77 to 80 % – Increase in growth, feed utilization	Ahmad et al. (2011)
Black cumin seeds (*Nigella sativa*)	Fingerling (40±5 g)	*A. hydrophila*	– Increase in survival with 24 % – Increased phagocytic activity by 6 %	Elkamel and Mosaad (2012)
Jackfruit (*Artocarpus integrifolia*) seed extract	Juvenile	*S. agalactiae*	– Increase in survival with 30 %	Toazza et al. (2013)

Table 1.4. Use of immunostimulants for health and growth promotion in tilapia culture (part 3)

Antagonist / active compound	Life stage / size	Disease causative agent	Effect	Reference
Vitamins and hormones/cytokines				
Vitamin E (Immunoton®)	Fingerling (28±5 g)	*A. hydrophila*	– Increase in survival with 50 %	Abdelkhalek et al. (2008)
Vitamin C (Levamisole®)	Fingerling (28±5 g)	*A. hydrophila*	– Increase in survival with 75 %	Abdelkhalek et al. (2008)
Vitamin C (Rovimix Stay-C® 35 DSM) (with β-glucan)	Fingerling (5.0±0.2 g)	*A. hydrophila*	– Increase in survival with 15 to 20 %	Barros et al. (2014)

Several other reasons for failure of the use of antimicrobial agents as disease control are (1) use of antimicrobials without improvement in farming practices / environment, (2) insufficient treatment duration, (3) utilization of subtherapeutic dosages, (4) use of antimicrobials of unsure/unproven quality, (5) lack of information concerning the fish stock (e.g. biomass) and (6) inadequate storage of chemicals (Yuan and Chen, 2012). Direct adverse effects resulting from the agent being in the environment or in the marketed product must also be considered. The emergence of antimicrobial resistant pathogens might pose a further risk to the health of human beings due to the ease of resistance-gene transfer between bacterial populations (Kesarcodi-Watson et al., 2008; Schwarz et al., 2001). Despite a lack of reports on adverse effects on human health from agents in aquaculture products, their presence has a major influence on market acceptability and on the economics of aquaculture (Karunasagar, 2012; Kesarcodi-Watson et al., 2008). For these reasons, nowadays several countries, governments and organizations have set tight legislations restricting the use of antibiotics in terrestrial as well as aquatic animal feeds. This ranges from certification of some drugs for use as in the case of several Asian countries to a total ban on the non-therapeutic use of any drug in the production of feed animals as in the case of the European Union (EU) and the Norwegian salmon industry (Kesarcodi-Watson et al., 2008). The use of antibiotics should only be considered a short-term and emergency type of solution. To avoid economic losses due to diseases and the stress of treatments themselves, a proactive strategy of prevention is strongly advised. The development of alternative treatments should focus on the search for microbial methodologies, which are based on natural processes ubiquitous in the environment (De Schryver et al., 2010; De Schryver and Vadstein, 2014; Defoirdt et al., 2007a).

Quorum sensing disruption

A number of Gram-negative pathogenic bacteria have a quorum sensing system that regulates the expression of their virulence (Bruhn et al., 2005). Quorum sensing (QS) is a mechanism by which bacteria coordinate the expression of certain genes in

response to the presence or absence of small signaling molecules (Defoirdt et al., 2007a). N-acyl-L-homoserine lactones (AHLs) are commonly produced and used by the bacteria as the QS signaling molecules (Antunes et al., 2010; Camilli and Bassler, 2006). For example, the production of AHLs was observed by the pathogen *E. tarda* isolated from diseased flounder and turbot (Han et al., 2010; Morohoshi et al., 2004) and *Pseudomonas* sp. isolated from diseased Nile tilapia (Chang et al., 2012). These findings suggest that some virulence factors of these pathogens are regulated by the QS system.

The disruption of bacterial quorum sensing, called quorum quenching, has been proposed as an anti-infective strategy and several techniques that could be used to disrupt quorum sensing have been investigated. These techniques comprise (1) the inhibition of signal molecule biosynthesis, (2) the application of quorum sensing antagonists (including natural occurring as well as synthetic halogenated furanones, antagonistic quorum sensing molecules and undefined exudates of higher plants and algae), (3) the chemical inactivation of quorum sensing signals by oxidised halogen antimicrobials, (4) signal molecule biodegradation by bacterial lactonases and by bacteria and eukaryotic acylases and (5) the application of quorum sensing agonists (Defoirdt et al., 2004). Chen et al. (2010) reported that recombinant AHL-lactonase from *Bacillus* sp., when co-injected with the fish pathogen *Aeromonas hydrophila* in common carp, decreased the mortality rate and delayed the time of death of the fish. Recently, Villamil et al. (2014) reported that the extracellular products of *Lactobacillus acidophilus* showed *in vitro* antibacterial capacity against pathogen *A. hydrophila* and *S. agalactiae* in Nile tilapia culture, and that viable *L. acidophilus* was able to disrupt quorum sensing activity by the inhibition of violacein production that is closely related to the mechanisms of N-acyl homoserine lactone disruption and quorum sensing regulation in *Chromobacterium violaceum* (Morohoshi et al., 2008). This suggests that *L. acidophilus* can be regarded as a potential biocontrol agent (Defoirt et al. 2004). Although the technique seems very promising, it will take a while before practical applications come within reach as very limited knowledge is available on the QS interference

(Tinh et al., 2008). Further research should be oriented on the establishment of the nature of the molecules responsible for the quorum quenching activity that makes it useful as a biocontrol agent.

Probiotics and prebiotics

The research of probiotics as an alternative biocontrol strategy is increasing with the demand for environment friendly aquaculture. Probiotics are defined as live microbial feed supplements that control and antagonist pathogens as well as promote the growth of the cultured organisms (Mohapatra et al., 2013; Verschuere et al., 2000). The probiotics most frequently used in fish, crustaceans and mollusks culture belong to the microaerophilic or facultative anaerobic genera *Carnobacterium, Bacillus, Lactobacillus, Lactococcus, Pediococcus*, *Enterococcus* (Gatesoupe, 2008; Mohapatra et al., 2013; Verschuere et al., 2000; Villamil and Novoa, 2009). Applications of several probiotic bacteria have indeed been found to improve survival and growth of Nile tilapia culture (Table 1.5).

The most relevant mechanisms of action of probiotic bacteria in wide aquaculture practices are enzymatic contribution to feed digestion / nutrients utilization (Merrifield et al., 2010; Musa et al., 2009; Verschuere et al., 2000), competition with pathogens (Balca´zar et al., 2006), antimicrobial activity (Picchietti et al., 2007; Picchietti et al., 2009; Villamil et al., 2003), quorum sensing disruption (Defoirdt et al., 2004) and production of inhibitory substances like bacteriocins and ribosomally synthesized antibiotic peptides that constitute one of the most potent weapons to fight pathogen infections (Desriac et al., 2010). Several studies also demonstrated beneficial effects of probiotics on the immune system (Dimitroglou et al., 2011; Nayak, 2010; Perez and Fontanetti, 2011), increase in stress tolerance (Arockia Raj et al., 2008; Rollo et al., 2006), as well as fish reproduction (Gioacchini et al., 2012; Giorgini et al., 2010) and fish development (Carnevali et al., 2004).

Table 1.5. Use of probiotics for health and growth promotion in tilapia culture

Probiotic	Life stage / size	Disease causative agent	Effect	Reference
Synbiotic *Streptococcus (Enterococcus) faecium* – *Lactobacillus acidophilus*	Juvenile (~0.1g)	No disease; normal culture	– Increase in survival with 17 to 20%	Lara-Flores et al. (2003)
Lactobacillus rhamnosus	Fingerling (60-70 g)	*E. tarda*	– Increase in survival with 25 to 40%	Pirarat et al. (2006)
Synbiotic *Lactobacillus acidophilus* - *Bacillus subtilis*	Fingerling (20-30 g)	*A. hydrophila* *P. fluorescens* *S. iniae*	– Relative level of protection (RLP) of 52 % – RLP of 51 % – RLP of 41 %	Aly et al. (2008)
Pediococcus acidilactici	Adult (~175 g)	Natural effects	– Increase in survival with 12 to 15%	Ferguson et al. (2010)
Lactococcus lactis	Fingerling (6.8±0.1 g)	*A. hydrophila*	– Increase in survival with 17 to 20% – Also increase in growth	Zhou et al. (2010)
Bacillus subtilis	Fingerling (30±5 g)	*Flavobacterium columnare*	– Amelioration of lesions	Mohamed and Refat (2011)

Besides probiotics, the applications of prebiotics have also been found to enhance growth and the health status of cultured animals. They are non-digestible dietary carbohydrates that escape digestion in the upper gastrointestinal tract but alter the bacterial composition of the gut by changing the type of substrate provided to the existing gut microbiota (Ringø et al., 2010). Compounds which have been shown to have prebiotic characteristics in aquaculture are fructo-oligosaccharides / FOS (Hui-Yuan et al., 2007), mannan-oligosaccharides / MOS (Dimitroglou et al., 2008; Sado et al., 2008), xylo-oligosaccharides (Xu et al., 2009), transgalacto-oligosaccharide / TOS, galacto-oligosaccharides (GOS), lactose, and inulin (Hoffmann, 2012). As such, prebiotics can be used as unique tools to create a gut microbiota with a controlled composition that can eventually be correlated with specific physiological conditions. This approach focuses on the beneficial activity of microorganisms already available in the aquaculture system, specifically the health-promoting microbial species already resident in the gastrointestinal tract of the aquatic animals (Ringø et al., 2010), such as *Lactobacillus* spp. and *Bifidobacterium* spp. (Manning and Gibson, 2004).

Other beneficial effects of a prebiotic are characterized by (i) a decrease in harmful bacteria in the gut of the host; e.g. decrease in growth of pathogenic *A. hydrophila, P. fluorescens and F. columnare* in Nile tilapia culture by the application of the commercial prebiotic Bio-Mos® containing MOS extracted from *S. cerevisiae* yeast (Abu-Elala et al., 2013), (ii) changes in digestive enzymes concentrations; e.g. increase in amylase, protease and lipase activity in rainbow trout juvenile by the application of the commercial prebiotic GroBiotic®-A (Azari et al., 2011), and (iii) improvement in host digestive system; e.g. increase in absorptive surface in the posterior gut region, microvilli density and microvilli length of rainbow trout, by the application of the prebiotic MOS (Dimitroglou et al., 2008)

Short Chain Fatty Acids (SCFAs)

Short chain fatty acids (SCFAs) are major end-products of fermentation of dietary compounds (Gibson and Roberfroid, 2008; Titus and Ahearn, 1988). In mammals, the SCFAs associated with specific antimicrobial activity are either simple monocarboxylic acids such as formic, acetic, propionic and butyric acid (Titus and Ahearn, 1988), or carboxylic acids bearing an hydroxyl group (usually on the α carbon) such as lactic (2-hydroxypropanoic), malic (hydroxybutanedioic), tartaric (2,3-dihydroxy-Butanedioic), and citric (2-hydroxy-1,2,3-propanetricarboxylic) acid (Dibner and Buttin, 2002; Mortensen and Clausen, 1996). Several investigations demonstrate the effect of organic acids on the reduction of bacterial counts in the stomach (Kluge et al., 2004) and the duodenum (Kirchgessner and Roth, 1991; Hebeler et al., 2000; Hellweg et al., 2006), while acid tolerant beneficial *Lactobacillus* spp. seem to be unaffected or may even be enhanced in numbers (Hellweg et al., 2006).

SCFA are known to inhibit the growth of some potentially pathogenic bacteria, such as *Salmonella* spp., *Vibrio* sp. and *Streptococcus* sp. (Defoirdt et al., 2007a; Ewing and Cole, 1994). The growth inhibitory effect of SCFAs is believed to be caused by the undissociated form of the acid which is able to penetrate through the bacterial cell membrane. Once inside, the acid releases protons (H^+) in the neutral cytoplasm decreasing the intracellular pH (De Schryver et al., 2010), forcing bacteria to redirect energy towards the efflux of the excess protons, thereby straining the cell metabolism leading to lower cell growth and even cell death (Kato et al., 1992) (Fig. 1.7).

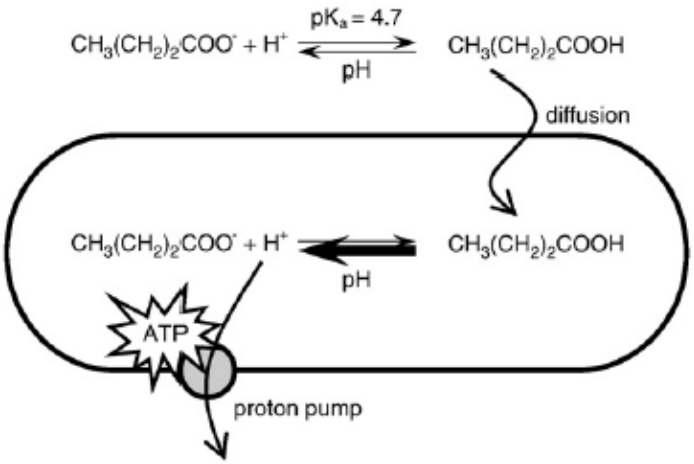


Figure 1.7. Mechanism of bacteriostatic activity of SCFAs (Defoirdt et al., 2009).

Even though the antimicrobial effect of organic acids is well understood, the explanation for their growth-promoting effects remains speculative (Lückstädt, 2007; Xie et al., 2003). SCFAs are known as an energy source for epithelial cells in the colon of mammals. In herbivorous mammals, i.e. ruminants, SCFAs production accounts for as much as 80 % of maintenance energy requirements (Engelhardt and Rechkemmer, 1985). Poultry and swine fed organic acid-supplemented diets have been reported to show improved feed intake, growth, feed utilization efficiency and health (Dibner and Buttin, 2002; Kluge et al., 2006; Partanen et al., 2002). In the field of aquaculture, the effects of SCFAs have not been studied to any great extent, but it has been shown that these substances can have growth promoting effects in several salmonids including Atlantic salmon, rainbow trout, and arctic charr, although some results are contradictory (Gislason et al., 1994; Lückstädt, 2007). In the case of Nile tilapia, citric acid (10^{-2} to 10^{-6} M) and lactic acid (10^{-2} to 10^{-5} M) that are added to experimental diet had stimulatory effects on the feeding response, as recorded by a function recorder (Xie et al., 2003). Another study reported potassium diformate (potassium salt of formic acid) as a growth promoter in Nile tilapia fingerlings, with an increase in weight gain of about 20 % (Ramli et al., 2005). Similar results were achieved by Zhou et al. (2008), where an increase in growth of up to 11.6 % was observed in hybrid tilapia (*O. niloticus* x *O. aureus*)

fingerlings (2.7 g initial weight) fed with potassium diformate at different concentrations (0 %, 0.3 %, 0.6 %, 0.9 % and 1.2 % dry feed weight) during a 56 days trial period. In contrast, Petkam et al. (2008) reported no significant improvement in the growth performance of Nile tilapia fingerling fed an acid blend Biotronic® A.S. (containing Ca-formate, Ca-propionate, Ca-lactate, Ca-phosphate and citric acid) or potassium diformate, respectively, at various dietary levels. Though there are only a limited number of published studies on the use of organic acids for growth promotion, feed efficiency as well as mineral absorption and disease prevention in tilapia aquaculture, results from those studies indicate promising potential and compel aqua feed manufacturers to consider using organic acids in their diets. However, for the application of FAs in practice, the pH of the intestine seems to be a limiting factor as it determines the ratio between the undissociated (or active) and the dissociated (or less active) form of the FAs and thus the effectiveness of the treatment (Dibner and Buttin, 2002). Furthermore, these FAs are water-soluble compounds and thus achieving an efficient uptake of the FAs by the treated animals is often problematic. As an alternative, polyhydroxyalkanoates (PHAs) are more attractive to be applied due to their water insolubility, thus increasing the uptake efficiency.

Polyhydroxyalkanoates (PHAs): production and degradation

Several studies have evaluated SCFAs as biocontrol agents in animal production, with emphasis on polyhydroxyalkanoates (PHAs), which are polymers of β-hydroxy SCFAs (De Schryver et al., 2010; Defoirdt et al., 2009). PHAs can be depolymerized by many different microorganisms that produce extracellular PHA depolymerases. PHAs can also be degraded upon passage through the animal's gastrointestinal tract and consequently might result in biocontrol effects similar to those described for SCFAs.

Polyhydroxyalkanoates (PHAs) are polyesters of various hydroxyalkanoates that are synthesized by many Gram-positive and Gram- negative bacteria from at least 75 different genera mainly under conditions of nutrient limitation and carbon

excess (Tian et al., 2009). More than 150 constituents of polyhydroxyalkanoates (PHAs) have been identified and characterized (Wang and Liu, 2014). These polymers are accumulated intracellularly as / discrete granules to levels as high as 90 % of the cell dry weight under conditions of nutrient stress and act as a carbon and energy reserve (Anderson and Dawes, 1990; Lenz and Marchessault, 2005).

PHAs producing bacteria can be divided in two groups based on the culture conditions required for PHA synthesis (Lee, 1996). The first group requires the limitation of an essential nutrient such as nitrogen, phosphorus, magnesium, potassium, oxygen or sulphur for the efficient synthesis of PHA from an excess carbon source. The second group of bacteria does not require nutrient limitation for PHA synthesis and can accumulate polymers during growth. *Alcaligenes eutrophus*, *Azotobacter vinelandii* and *Pseudomonas oleovorans* and many other bacteria belong to the first group, while some bacteria such as *Alcaligenes latus* and recombinant *E. coli* and *Klebsiella* strains harbouring the PHA synthesis genes belong to the second group (Defoirdt et al., 2009).

PHAs can be divided into three subgroups based on the number of carbon atoms in the monomer unit incorporated into the polymers. PHAs containing up to five carbon atom (C5) monomers are classified as short-chain-length PHA (scl-PHA). PHA with C6 - C14 and > C14 monomers are classified as medium-chain-length (mcl-PHA) and long-chain-length (lcl-PHA) PHA (Wu et al., 2009) (Fig. 1.8). Because of inherent biodegradability, PHAs have attracted the world-wide attention of scientists and researchers as an environment-friendly alternative to the conventional petroleum-based polymers and applications of PHAs have been reported in packaging industry, agriculture, automotive sector, food industry and medical implantation devices (Kalia et al., 2011).

PHAs differ from synthetic polymers because of their biodegradability by depolymerase enzymes in bacteria. The PHA degradation pathway in PHA accumulating bacteria begins with the depolymerisation of PHA to β-

hydroxyalkanoate monomers by intracellular PHA depolymerase enzymes (Kadouri et al., 2005).

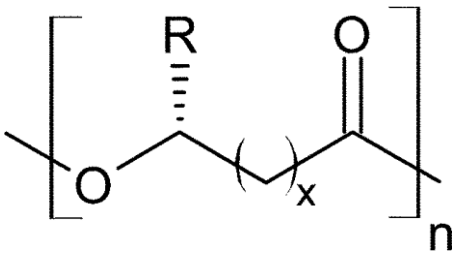


Figure 1.8: General structure of polyhydroxyalkanoates, R is variable. When X = 1, R = CH_3, the monomer structure is 3-hydroxybutyrate and the polymer is poly-3-hydroxybutyrate (poly-β-hydroxybutyrate) (Wu et al., 2009).

Different aerobic and anaerobic microorganisms isolated from a variety of ecosystems are able to degrade extracellular PHAs. Depending on the pH, temperature and polymer crystallinity, the degradation of PHAs occur via enzymatic or chemical hydrolysis. Characteristics such as stereoregularity, crystallinity, monomeric composition and accessibility of the polymer surface affect the biodegradability by the microorganisms (Defoirdt et al., 2009). PHAs can also be degraded upon passage through the gastrointestinal tract of animals and consequently adding these compounds to the feed might result in biocontrol effects similar to those described for SCFAs. As PHAs can be degraded into SCFA β-hydroxy (i.e. β-hydroxybutyrate), the potential of β-hydroxybutyrate and its natural polymer poly-β-hydroxybutyrate (PHB) as biocontrol agents in aquaculture practices have been intensely investigated.

β-hydroxybutyrate

β-hydroxybutyrate (β-HB) has important roles in mammalian health and disease (Dedkova and Blatter, 2014). In mammals, β-HB is a metabolic intermediate that constitutes up to 70 % of ketone bodies. The term "ketone bodies" refers to three molecules: (1) β-HB, (2) its dehydrogenated counterpart acetoacetate (AcAc), and (3) the decarboxylated AcAc, acetone. The β-HB is produced in liver mitochondria during ketogenesis, the process by which FAs released from adipose tissue are

transformed into AcAc and β-HB (Dedkova and Blatter, 2014) (Fig. 1.9). The process can occur in response to unavailability of blood glucose (Robinson and Williamson, 1980), where the production of ketone bodies is then initiated to make available energy primarily from fatty acids. The ketone bodies are then transported by the blood to the extrahepatic tissues, where they are oxidized via the tricarboxylic acid (TCA) cycle to provide the energy required by tissues such as skeletal and heart muscle. Studies with rats indicated that β-HB was shown to be one of the main precursors, next to acetate and butyrate, of the *de novo* synthesis of lipids in colonic epithelial cells in rats (Zambell et al., 2003). The net effect is a greater potential for ATP production, suggesting β-HB as the most efficient fuel in the heart (Dedkova and Blatter, 2014; Kashiwaya et al., 1994; Sato et al., 1995). β-HB can also serve as building block for the synthesis of fine chemicals such as vitamins, antibiotics, aromatics and pheromones (Chiba and Nakai, 1985; Seebach et al., 2001). It has been patented as human nutritional and dietary supplement for the purpose of seizure control, metabolic disease control, reduction of protein catabolism, appetite suppression, increased cardiac efficiency, treatment of diabetes, and treatment of effects of neurodegenerative disorders and epilepsy (Martin et al., 2000). Recently, Dedkova and Blatter (2014) reported that in mammals a mild elevation in β-HB levels can modulate the important signaling cascades involved in cell growth, cell proliferation and defense against oxidative stress, while its polymer PHB has proved to be involved in regulation of protein channels in plasma membrane and mitochondria.

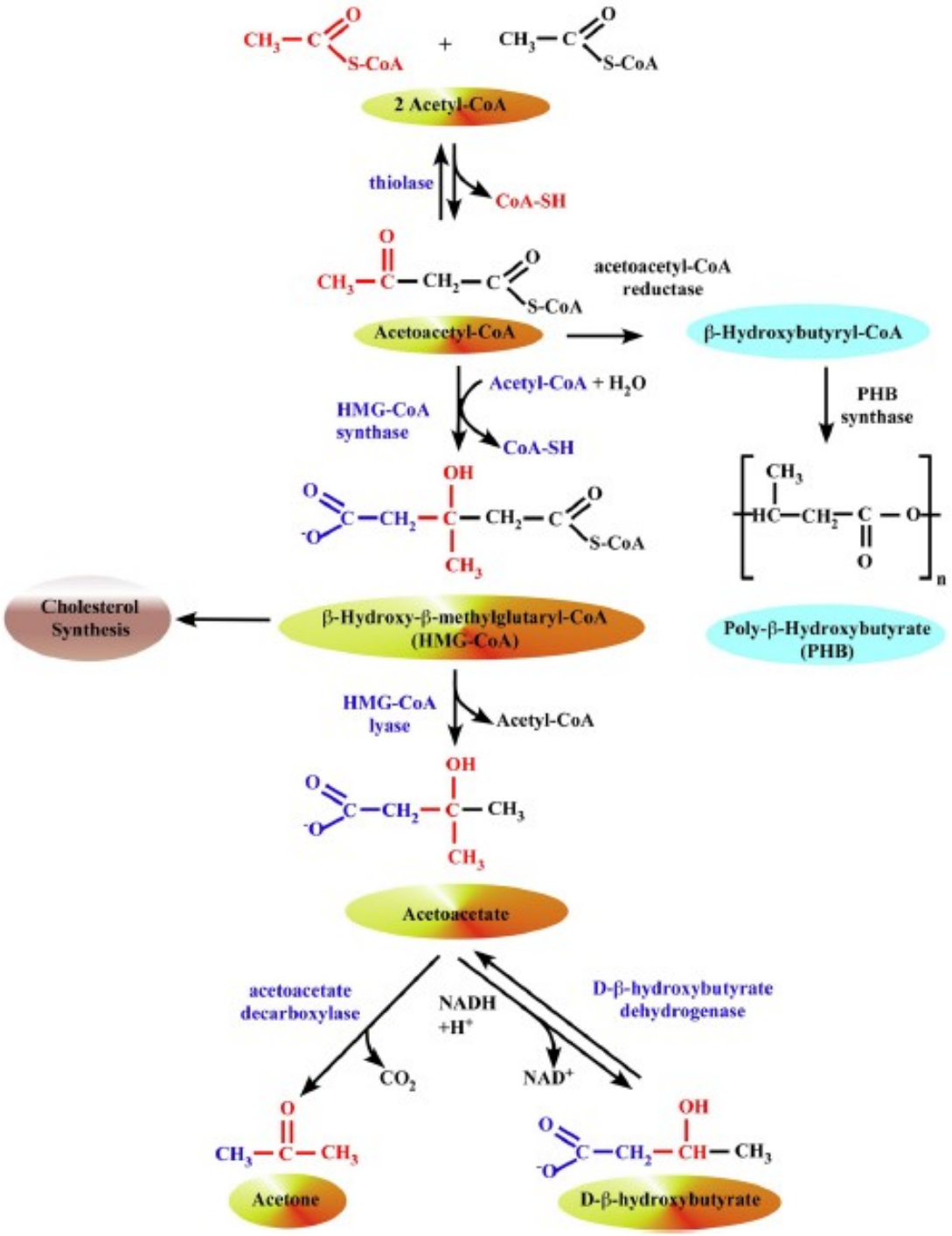


Figure 1.9. **Synthesis of β-HB in the formation of ketone bodies in liver mitochondria; reproduced from Dedkova and Blatter (2014).** The synthesis of β-HB begins with the condensation of two molecules of acetyl-CoAtoformacetoacetyl-CoA, the parent of the three ketone bodies, by a ketothiolase enzyme. In prokaryotes, this intermediate is subsequently reduced with NADPH to hydroxybutyryl-CoA by acetoacetyl-CoA reductase, and hydroxybutyryl-CoA may then be polymerized to form PHB by the enzyme PHB synthase. In eukaryotes, 3-hydroxy-3-methyl glutaryl-CoA synthase (HMG synthase) catalyzes the condensation of acetoacetyl-CoA with a third acetyl-CoA to form β-hydroxy-β-methylglutaryl-CoA (HMG-CoA). The enzyme HMG-CoA lyase then catalyzes the decomposition of HMG-CoA to form acetoacetate and acetyl-CoA, and acetoacetate is further reduced with NADH by phosphatidylcholine-dependent mitochondrial D-β-hydroxybutyrate dehydrogenase to form β-HB.

In mammals and other higher vertebrates, during the ketone body metabolism, β-HB can only be produced from FAs if the enzyme β-HB dehydrogenase (HBDH) is available (Fig. 1.9). In fish, activity of HBDH has been detected in freshwater teleost species, including the northern brown bullhead *Ictalurus nebulosus*, northern pike *Esox lucius*, black crappie *Pomoxis nigromaculatus*, and goldfish *Carassius auratus*, and also marine teleost species, including alewife *Alosa pseudoharengus*, smelt *Osmerus mordax*, and mummichog *Fundulus heteroclitus*, with the highest activity observed in the liver and kidney of fresh water teleosts and in the brain and liver of the marine teleosts (Leblanc and Ballantyne, 1993; 2000). The role of cytosolic form of HBDH in mammalian ruminants –whose nutrients were obtained mostly from plant carbohydrates– has been attributed to the use of butyrate from microorganisms as a carbon source for ketone body formation (Watson and Lindsay, 1972). Consequently, it has been hypothesized that in fishes capable of digesting plant carbohydrates, a system similar to that of ruminant mammals may prevail (Ballantyne, 2014), and this could be the case for tilapia. β-HB formation from butyrate has been demonstrated in liver slices of rainbow trout (*Salmo gairdneri*) and eel (*Anguilla australis*), contributing to 6 % of the measured total ketone bodies (AcAc and β-HB) in the liver slices (Phillips and Hird, 1977). β-HB has also been detected in the plasma of the two species at levels similar to those of mammals of 60-70 % of total blood ketone bodies (Phillips and Hird, 1977). However, further studies are needed to demonstrate the utilization of β-HB and to elucidate the role of ketone bodies in fish.

Poly-β-hydroxybutyrate (PHB)

Like their petrochemical counterparts, PHAs are moldable, water-insoluble, thermoplastic polymers. PHB (also known as poly-β-hydroxybutyrate), the most common PHA, is a stiff, high-melting-point aliphatic polyester similar to many industrial polyolefins (polyethylenes, polypropylenes), that can be used as an alternative source of plastics (Byrom, 1987). It is a biopolymer consisting of linear

chains of β-HB. Three groups of PHB with different numbers of β-HB units and with different functions have been discovered: (i) high molecular weight storage PHB consists of 10,000 to > 1,000,000 β-HB residues (storage PHB), (ii) low molecular weight PHB with medium–chain length consisting of 100–300 residues (oligo-PHB), and (iii) short-chain conjugated PHB in which low numbers of β-HB residues (≤ 30) are covalently linked to proteins (see Reusch 1992, 2012, 2013 for comprehensive reviews). Storage PHB was first discovered in granular inclusion bodies (termed carbonosomes) within cytoplasmic granules of *Bacillus megaterium* in 1925, and later in a wide variety of archaea and eubacteria, principally those that inhabit soil and water ecosystems (Anderson and Dawes, 1990; Nuti et al., 1972; Poli et al., 2011). PHB produced by these prokaryotes when nutrients such as nitrogen and phosphorous source are available in limiting concentrations in the presence of excess carbon source (Anderson and Dawes, 1990; Reusch, 2012).

Even though there are more than 250 different microorganisms synthesizing PHAs, only several of these, such as *Alcaligenes eutrophus* (Kim et al., 1994), *Alcaligenes latus* (Yamane et al., 1996), *Azotobacter vinelandii* (Page and Knosp, 1989), methylotrophs (Kim et al., 1996), *Pseudomonas oleovorans* (Brandl et al., 1988) and recombinant *Escherichia coli* (Lee and Chang, 1994; Lee et al., 1997) are suitable for the production of PHAs to a high concentration with high productivity. Biodegradable plastics have been available for many years on the market; however, their high cost has meant they have not replaced the traditional non-degradable plastics. Up until now, researchers continue to work on the optimization of PHB production by microorganisms through evaluation of the physical and environmental factors that lead to the maximization of PHAs production using agro-industrial waste products as cheap carbon sources, and scaling up the product as a step to be used in large scale industry for its high economic and commercial value and its value as a product that is safe for the environment.

Use of PHB as biocontrol agent in aquaculture

In the early investigation of the potential use of PHB for aquaculture applications, Defoirdt et al. (2007b) showed that PHB increased survival and growth of starved *Artemia* nauplii and also protected the nauplii culture from *V. campbellii* infection. It was hypothesized that the higher growth of *Artemia* nauplii and protection against *V. campbellii* following PHB supplementation is due to the release of β-HB and production of ketone bodies (Defoirdt et al., 2007b). In order to evaluate the potential use of PHB as growth modulator, De Schryver et al. (2010) replaced European sea bass diet partially with PHB at a 2 %, 5 % and 10 % inclusion level. Their findings suggested that PHB could serve as an energy source in sea bass juveniles (as for a diet with 100 % PHB survival was considerably higher than for starved animals) and significantly increased growth rate and decreased FCR when present at 2 % and 5 % in the diets. A decrease of the gut pH from 7.7 to 7.2 was observed, suggesting that PHB can be degraded in the gut, leading to an increased production of acidic compounds, likely β-HB. It is reported that the highest bacteria range-weighted richness in the fish intestine was observed in the 2 % and 5 % PHB treatments, and that higher dietary PHB levels induced larger changes in the bacterial community composition (De Schryver et al., 2010).

Another study by Nhan et al. (2010) investigated the effects of feeding enriched *Artemia* nauplii with PHB and/or highly unsaturated fatty acids (HUFAs) on the growth performance of giant freshwater prawn (*Macrobrachium rosenbergii*) larvae. Their results revealed that feeding the larvae with PHB containing *Artemia* nauplii significantly increased survival as well as larval development. Also, PHB significantly decreased the number of *Vibrio* spp. and total bacterial counts when compared to a control treatment, indicating that the PHB addition had a growth-inhibitory influence towards these potentially pathogenic microorganisms. PHB was also tested in Siberian sturgeon (*Acipenser baerii*) fingerlings (Najdegerami et al., 2012). In their study, PHB affected the gut microbial species richness and diversity; however, unlike studies of European sea bass and giant freshwater

prawn, no growth modulation was observed. The effects of feeding *Artemia* nauplii enriched with or without PHB and/or HUFA on sturgeon larvae were also investigated (Najdegerami et al., 2013). The results showed that PHB and PHB+HUFA supplementation decreased the growth performance of the larvae. In addition, PHB decreased the survival when larvae were challenged in different salinities and ammonia stress levels. It is suggested that the effects of PHB can be species specific and thus the optimal PHB doses for bio-encapsulation into *Artemia* remain to be determined for application at the earliest larval stages of sturgeon.

Sui et al. (2012) investigated the protective effect of PHB on Chinese mitten crab (*Eriocheir sinensis*) zoea larvae challenged with *V. anguillarum* at a final concentration of 10^5 CFU mL^{-1}. PHB was delivered to the larvae through rotifer and *Artemia* bioencapsulation, which were added to the culture water 24 h prior to challenge, upon challenge and 24 h after challenge. The results illustrated that PHB could enhance the survival and growth of unexposed Chinese mitten crab larvae. Moreover, PHB protected the larvae from the pathogen as the larvae fed PHB-enriched live food showed the highest survival and development rate in all challenged groups. Furthermore, larval performance was the best when PHB was delivered to the larvae 24 h before challenge. These results indicate that PHB can be used as part of an effective strategy to protect Chinese mitten crab larvae from *V. anguillarum* infection resulting in higher survival and better growth, especially when applied before the challenge.

More recently, Thai et al. (2014) investigated the effect of administering PHB-accumulated *Alcaligenes eutrophus* H16 (containing 10 % or 80 % PHB on dry weight) on the culture performance of larvae of the giant freshwater prawn. It was found that feeding prawn larvae with *Artemia* nauplii enriched in a medium containing 100 and 1,000 mg L^{-1} *A. eutrophus* containing 80 % PHB significantly increased the survival with about 15 % and the development of the larvae with a larval stage index of about 1 as compared to feeding non-enriched *Artemia*. The survival of the larvae also significantly increased with about 35 % in case of a

challenge with *V. harveyi*. Their findings emphasized that PHB supplemented in a bacterial carrier (i.e. amorphous PHB) can increase the larviculture efficiency of giant freshwater prawn similar to supplementation of PHB in powdered form (i.e. crystalline PHB).

All previous studies suggest the potential of PHB as a biocontrol agent, which may serve as an alternative to antibiotic treatments for a more sustainable aquaculture. Furthermore, PHB can be completely degraded aerobically and anaerobically by microorganisms (Miller and Williams, 1987; Pişkin, 1995). The biodegradability of PHB may thus also be considered as an attractive property of PHB with respect to sustainable aquaculture. The biodegradation of PHB and its composites in natural ecosystems, such as soil, compost, and bodies of water, was described in a number of publications (Iordanskii et al., 2014; Lenz and Marchessault, 2005; Mergaert et al., 1992). The end products of PHB biodegradation in aerobic environments are carbon dioxide and water, while methane is also produced in anaerobic conditions (Song et al., 2009). Unlike in agriculture / livestock production, the production of methane gas due to the anaerobic biodegradation of aquaculture waste derived from PHB-supplemented system will have very little impact, if any, on the environment due to the low level of PHB supplementation in fish diets (ranging from 2 to 5 % PHB diet in the previous studies), considering the high cost of PHB production (Choi and Lee, 1997; 1999; De Schryver, 2010).

Research objectives

The general objective of this study was to explore the full potential of PHB as an ecologically and economically sustainable alternative as growth modulator and anti-infective strategy for aquaculture production. Despite the demonstrated growth modulation and protection against bacterial infections in different aquatic animals, the mechanisms of action of PHB or its fate within the animal are far from understood. Previously, the beneficial effect of PHB on fish culture performances has been studied in European sea bass culture – carnivorous; marine cold-water

species – and Siberian sturgeon culture – carnivorous; freshwater cold-water species –; both are species that are high at the trophic level. PHB has not been tested on fish species that feed generally on a low trophic level, and which can rely from food items from different trophic levels to a varying extent and thus are more flexible in terms of feed ingredient use and might benefit more from dietary PHB supplementation. As for this, Nile tilapia – omnivorous; freshwater warm-water species – is chosen as the experimental animal in this study. This study evaluates the application of PHB and its beneficial effects for tilapia culture at different developmental stages of tilapia (juvenile, fingerling), in different culture conditions (open system vs. gnotobiotic/closed system) and identifies possible mechanisms of action responsible for the beneficial effects of PHB on the fish growth and health promotion. Figure 1.10 illustrates how the different chapters in this manuscript contribute to knowledge in those two fields.

Thesis outline

The effects of PHB on growth parameters, fish composition and digestive enzyme activities of Nile tilapia juvenile culture are evaluated in **Chapter 2**. As the gut microbial community (MC) is hypothesized to play a significant role in the nutrient digestion and metabolism, the effects of PHB on the functional physiological profiles of the tilapia gut MC are evaluated in **Chapter 3**. Using the Biolog Ecoplate™ assay, it was targeted to apply the gut microbiota's functional physiological profile to provide an understanding of the mechanism of action of PHB on fish growth modulation in terms of microbial nutrient digestion. **Chapter 4** then describes the investigation of the compartmental distribution of PHB throughout the fish body after ingestion using the stable isotope ^{13}C as a PHB tracer. Based on the obtained results hypotheses were formulated about the PHB metabolism / utilization in the fish body and the possible mechanism of action of PHB in fish growth modulation in terms of fish physiology. In addition to the study of PHB on tilapia growth modulation, the effect of PHB on tilapia disease

resistance is evaluated in **Chapter 5**. The survival performance of fish larvae supplemented with PHB was studied in a gnotobiotic bacterial challenge test. Here, the effect of PHB on larvae resistance towards pathogen infection is discussed. The last chapter (**Chapter 6**) provides an overview and discussion of the research outcomes from previous chapters and formulates recommendations for future research.

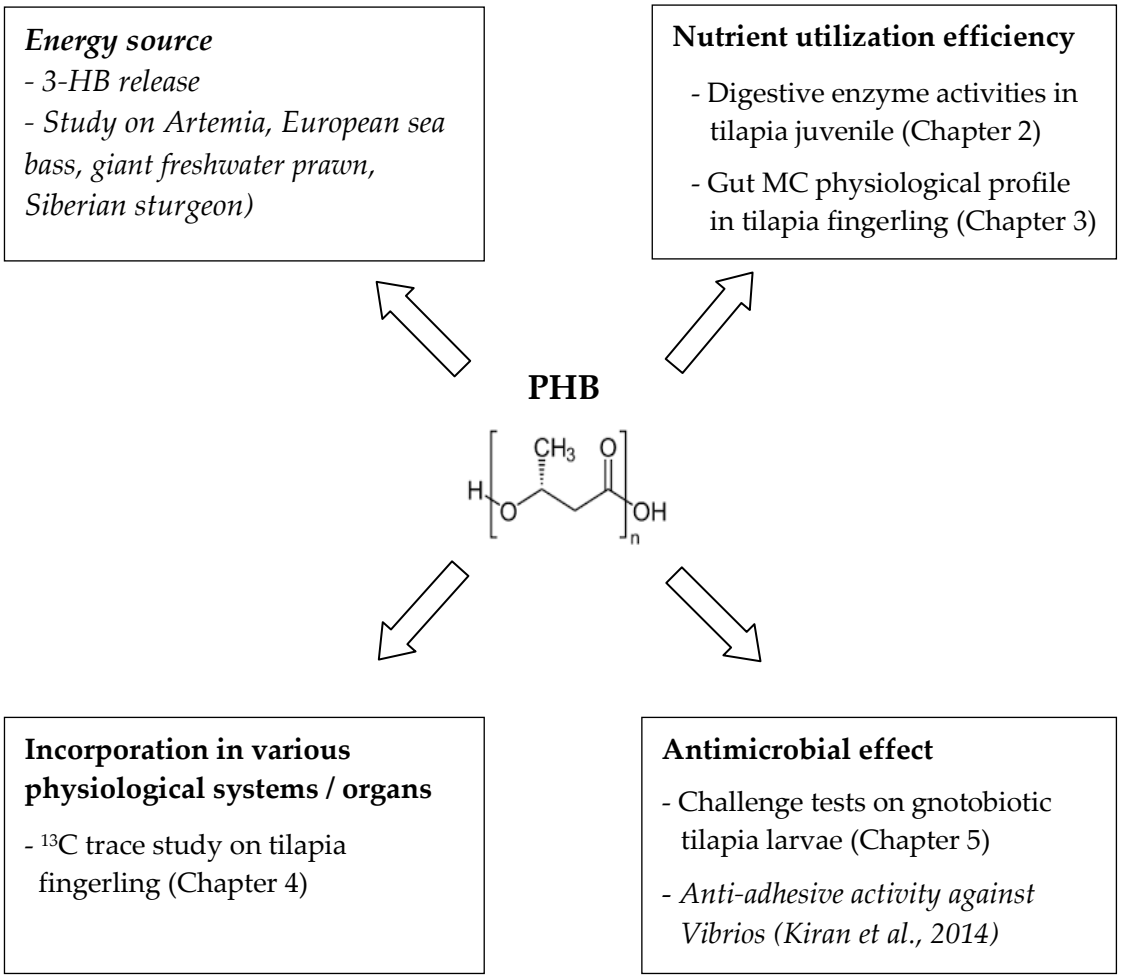
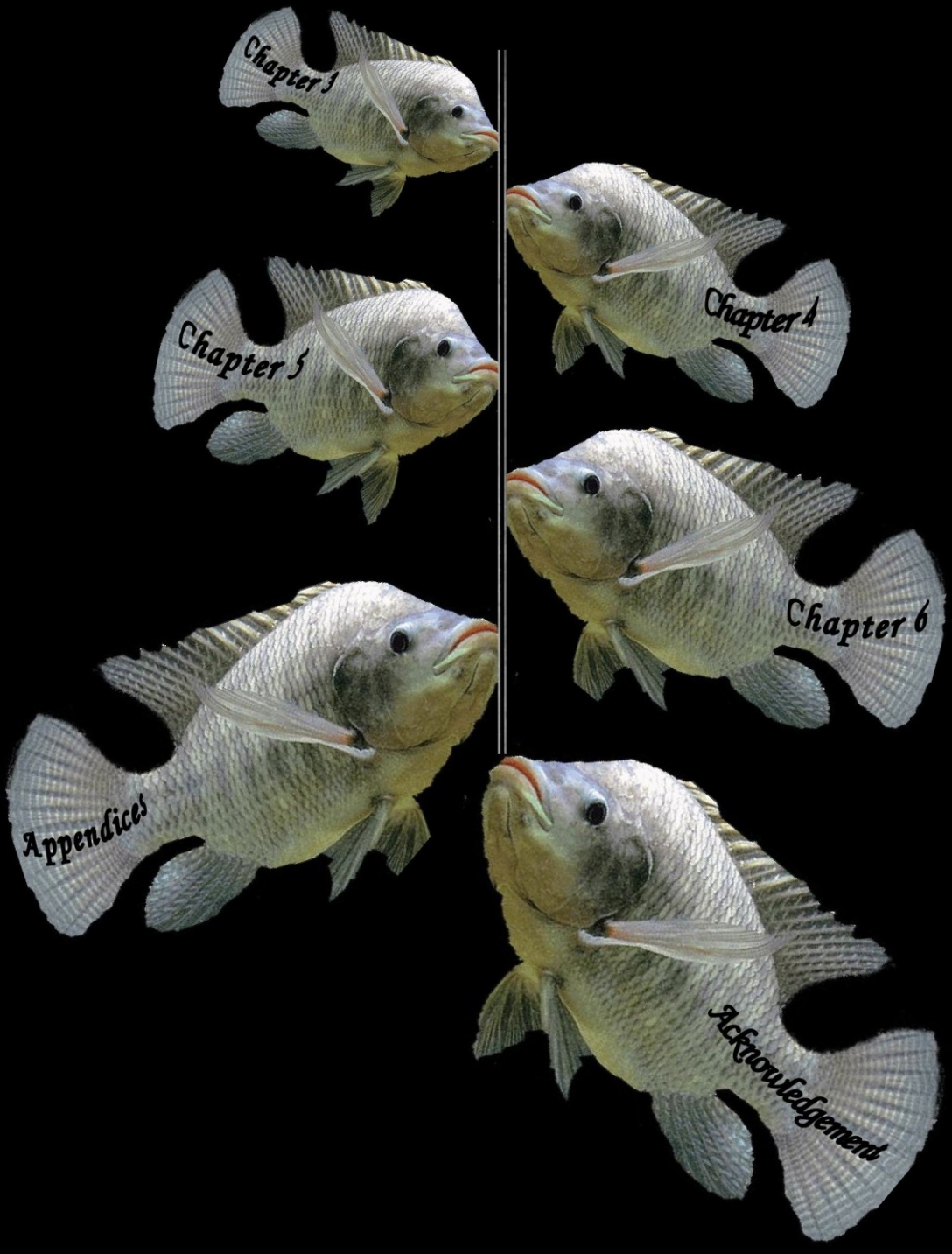


Figure 1.10. Overview of possible roles of PHB in the enhancement of aquaculture performance and the coverage of these aspects in the chapters of the present manuscript. The italic letters indicate information / results from other studies (not covered in the thesis).

Chapter 2

Poly-β-hydroxybutyrate supplementation in Nile tilapia juvenile culture



Chapter 2

Poly-β-hydroxybutyrate supplementation in Nile tilapia juvenile culture

Redrafted after:

Situmorang, M.L.[a,b], Dierckens, K.[a], De Schryver, P.[a], Bossier, P.[a], 2015. Poly-β-hydroxybutyrate supplementation in Nile tilapia *Oreochromis niloticus* juvenile culture. Aquaculture, submitted.

[a] *Laboratory of Aquaculture & Artemia Reference Center (ARC), Ghent University, Belgium*

[b] *School of Life Sciences and Technology, Bandung Institute of Technology, Indonesia*

Abstract

The contribution of poly-β-hydroxybutyrate (PHB) provided in different formulations to the growth stimulation and protection against bacterial infections has been studied in several aquatic animal species. This study investigated the effect of crystalline PHB, directly included in the formulation of a semi-purified diet, on the survival and growth parameters, digestive enzymes activity, and body composition of juvenile Nile tilapia (*Oreochromis niloticus*). Dietary PHB supplementation over a 28 days feeding period resulted in no significant difference in the mean final body weight (258 to 284 mg), when compared to the control group (218 mg). Digestive enzyme lipase activity was increased with about 20 to 40 % by the dietary PHB supplementation. This higher lipase activity might have led to the significant increase in the total lipid content with about 10 % in the PHB treatment groups. Dietary PHB resulted in increasing contents of total (n-6) fatty acids (FAs), total monounsaturated FAs and total saturated FAs with about 15 % DW, 15 % DW and 20 % DW, respectively, as compared to the control. However, the composition of the FAs groups in the total lipid was similar among various PHB treatments. This study suggests that PHB may potentially improve the growth of Nile tilapia, possibly by the stimulation of intestinal lipid digestion, absorption and deposition. We hypothesize that the altered lipid utilization may have resulted from diet-induced alterations in the GI microbiota composition. Future studies on the host microbiota-diet interactions are expected to contribute to a more advanced knowledge of the possible mechanisms of action of PHB in the alteration of nutrient utilization by the host, which could potentially result in growth enhancement in aquaculture practices.

Introduction

The expansion of aquaculture production and intensification of culture systems goes hand in hand with an increase in bacterial and parasitic fish disease outbreaks (Miranda et al., 2013). Under intensive culture conditions, fish are exposed to numerous stressors that can interfere with their immune response and resistance to infectious microorganisms in the culture environment. Antibiotics, disinfectants and parasiticides have been commonly applied to improve the health status of the cultured organisms and to prevent or treat bacterial and parasitic diseases in fish and crustacean cultures. However, the excessive use of these antimicrobials leads to multiple resistance in pathogens (Defoirdt et al., 2007a) and negatively affects animal and human health as well as the aquatic environment (Cabello et al., 2013; Rico and Van den Brink, 2014; Sapkota et al., 2008). Therefore, such products are being withdrawn from the global market. Consequently, different alternative biocontrol measures including the use of probiotics (Nayak, 2010; Tinh et al., 2008; Verschuere et al., 2000) and quorum sensing disruption (Defoirdt et al., 2004; Defoirdt et al., 2006a; Defoirdt et al., 2011) have been developed to control bacterial infections and to achieve an environment friendly sustainable aquaculture industry.

Also short-chain fatty acids (SCFAs) have been suggested as a bio-control measure in aquaculture (Defoirdt et al., 2006b; Defoirdt et al., 2007a; Defoirdt et al., 2009). However, their main limitation for application in aquaculture is their polar nature making them highly soluble and easy to diffuse in the culture water. This results in a low uptake efficiency of the SCFAs by the animal and thus demands for higher concentrations to be effective at the animal level (Defoirdt et al., 2011). A solution has been provided in the form of the bacterial polymer PHB, which is water insoluble and biologically degradable (Anderson and Dawes, 1990; Doyle et al., 1991). It serves as a carbon reserve and intracellular energy source for different species of bacteria (De Schryver et al., 2010; Defoirdt et al., 2011; Tokiwa and Calabia, 2007).

Several studies have confirmed the protective role of PHB in the host against bacterial infections (Defoirdt et al., 2007b; Sui et al., 2012; Thai et al., 2014), but interestingly enough an increased growth performance in crustaceans and fish has also been described. The use of PHB, both in particulate and bacterial form, resulted in a significant increase in survival of starved *Artemia* nauplii (Defoirdt et al., 2007b). Nhan et al. (2010), then again, found an increased growth performance and survival in giant freshwater prawn (*Macrobrachium rosenbergii*) larvae fed with PHB-enriched *Artemia*. In another study, Sui et al. (2012) have also found an enhanced growth and survival effect of PHB-enriched rotifer and *Artemia* on the larvae of the Chinese mitten crab (*Eriocheir sinensis*). And most recently, it has been described that the supplementation of PHB in a bacterial carrier (i.e. amorphous PHB) was found effective to support the growth and development of *M. rosenbergii* larvae (Thai et al., 2014). The beneficial effects of PHB dietary supplementation on growth performance have also been described for fish. Examples are European sea bass (*Dicentrarchus labrax*) juveniles and Siberian sturgeon (*Acipenser baerii*) fingerlings (De Schryver et al., 2010; Najdegerami et al., 2012).

In this study, the effect of amorphous PHB administered via a semi-purified diet to Nile tilapia (*Oreochromis niloticus*) at the early juvenile stage was investigated in terms of survival and growth performance, and the digestive enzyme activities. Based on the current knowledge and the results of this study, suggestions for future studies to further evaluate the mechanisms of action of PHB are made.

Materials and Methods

Experimental setting

Nile tilapia juvenile were produced and reared on commercial diet at the Aquaculture and Fisheries Group (AFI) of Wageningen University and Research Centre, the Netherlands. Juvenile were collected and acclimated in a 50 L aquarium for 14 days prior to stocking. During this period, they were fed the experimental basal diet (without PHB supplementation) three times daily to apparent satiation.

At the end of the acclimation period, fish with an average weight of 26.4±2.4 mg were randomly distributed in 12 rectangular aquaria of 38 L (50 fish per aquarium) with a flow-through system supplied with dechlorinated, heated tap water at a water renewal rate of 38 L/5 L h^{-1} hour, results in a hydraulic retention time of 7.2 h. During the feeding experiment, water temperature was maintained at the level of 28±1 °C with pH level ranging from 8.2 to 8.8. NH_4^+, NO_2^-, and NO_3^- level never exceed 0.05 mg L^{-1} NH_4-N, 0.5 mg L^{-1} NO_2-N and 2.5 mg L^{-1} NO_3-N, respectively. The experiment was carried out under a 16:8 h light:dark light regime. Water flow rate was checked and adjusted daily to ensure proper water exchange. In order to prevent feed loss during the feeding period by water exchange, the flow through system was only activated during the dark period.

Experimental diets and feeding regime

A basal semi-purified diet (control diet) was formulated to contain approximately 450 g kg^{-1} crude protein (Balarin and Halfer, 1982) and 150 g kg^{-1} lipid (Ng and Chong, 2004) based on the feedstuff values reported in NRC (1993) (Table 2.1). The analyzed crude protein level in the basal diet, however, was higher than expected (52.4 % DM instead of 45 % DM). The PHB experimental diets were the basal diet supplemented with three levels of crystalline PHB (5, 25, and 50 g kg^{-1} diet; Goodfellow Cambridge Limited, Huntingdon, England), at the expense of the α-cellulose. The moist mixture was extruded through a 3-mm diameter meat grinder (Hobart Corp., Troy, Idaho, USA). The resulting moist pellets were air-dried at room temperature to a moisture content of about 100 g kg^{-1}. Pellets were ground into small pieces, sieved to obtain appropriate sizes (300-500 μm) and stored at −20 °C until used. Fishes were fed daily at a fixed feeding level of 20 % on fish wet body weight (BW) (Ng and Romano, 2013) in three equal meals given every 4 hours between 09:00 and 17:00. The feed amount was adjusted daily based on mortality and weekly based on the average weight of the fish in the tank. Each experimental diet was tested in triplicates.

Table 2.1. Formulation of basal semi-purified diet (control diet) for Nile tilapia juvenile

Ingredients	g kg^{-1}
Carboxymethyl cellulose (Sigma)	20
Vitamin C (Stay-C 35 % VDS)	0.6
L-methionine (Sigma)	5
Vitamins + Minerals mix (VDS)	12.5
L-lysine (Sigma)	15
Choline chloride	1.8
α-cellulose	50
Corn meal (Bio Planet)	100
Fish herring meal (VDS)	200
Corn gluten meal (Sigma)	500
Fish oil (VDS)	20
Soybean oil	75
Vitamin E (95 %)	0.1
Analyzed nutrient content	g kg^{-1} DM
DM (g kg^{-1})	922
Crude protein	524
Crude fat	146
Ash	54
Total carbohydrates*	276

*Calculated as follows: total carbohydrates (starch, free sugars and NSP) = 1000 - (crude protein + crude fat + ash)

Fish survival and growth parameters

Six juveniles from each replicate tank were randomly collected every week minimally 14 h after the last feeding to monitor the growth performance. The average weight gain of the fish in a tank over the 28 days was calculated by subtracting the weight of the fish sampled on the final day of the experiment with the average weight of the fish as measured at the beginning of the experiment, and then taking the average. The specific growth rate (SGR) was calculated as follows:

$$\text{SGR (\% body weight gain per day)} = \left[\frac{(\ln W - \ln W_0)}{t}\right]$$

where W is the average body weight after 28 days, W_0 is the average initial body weight, and t is experimental period (28 days). The same approach was used to

calculate the feed conversion ratio (FCR), expressed as the feed consumption over the weight increase of the fish per treatment. Fish survival for individual treatments was determined as the number of surviving fish at the end of experimental period relative to the number of fish at the beginning of the experimental period.

Fish sampling and analyses

Assessment of digestive enzymes activity

Whole-body homogenates were used instead of organ-specific (i.e. intestine) homogenates to provide enough wet sample for enzymatic assays due to the small size of fish. Fish samples were taken minimally 14 h after the last feeding. Three fishes from each replicate tank were randomly sampled, euthanized with an overdose of Tricaine mesylate (0.1 % Tricaine methanesulfonate, MS-222; Sigma Chemical Co., St Louis, USA) and stored at -80 °C until homogenization. Samples were homogenized in 50 mM Tris-HCl buffer at 5:1 ratio (pH 7.5) in an electric homogenizer (Heidolph, Instruments Switzerland). All these processes were performed on ice. The homogenate was centrifuged at 10,000 *g* for 20 min at 4 °C and the supernatants collected and stored in small aliquots (100–200 μL) at −80 °C until the spectrophotometric assays of digestive enzyme activities. Lipase activity was quantified using 10 mM p-nitrophenol myristate as a substrate in 0.25 M Tris-HCl (pH 9.0). After 15 min of incubation at 25 °C, the p-nitrophenol released was monitored spectrophotometrically at 405 nm, using a standard curve of absorbance at wavelength of 405 nm against different p-nitrophenol concentrations (0–500 μmol). One unit of lipase activity is defined as the amount of substrate (μmol) hydrolyzed per minute per mg protein at 25 °C (Iijima et al., 1998). Trypsin activity was quantified using N-*α*-benzoyl-dl-arginine p-nitroanilide (BAPNA) as a substrate in 50 mM Tris-HCl and 20 mM $CaCl_2$ buffer (pH 8.2). After 15 min of incubation at 25 °C, the BAPNA released was monitored spectrophotometrically at 407 nm, using a standard curve of absorbance at wavelength of 407 nm against different BAPNA concentrations (0–500 μmol). One unit of trypsin activity

represents 1 μmol equivalent liberated BAPNA per min at 25 °C (Holm et al., 1988). Pepsin activity was quantified using 2 % hemoglobin in 0.06 N HCl as a substrate (Cahu and Zambonino Infante, 1994) and tyrosin as a standard. After 10 min of incubation at 37 °C, the tyrosin released was monitored spectrophotometrically at 280 nm, using a standard curve of absorbance at wavelength of 280 nm against different tyrosin concentrations (0–500 mg L^{-1}). One unit of pepsin activity is defined as the amount of enzyme required for the formation of 1 mg of tyrosin per min at 25 °C. Amylase activity was determined as illustrated by Najdegerami et al. (2013) using 0.3 % soluble starch as substrate dissolved in NaH_2PO_4 buffer (pH 7.4). After 30 min of incubation at 37 °C, the starch hydrolyzed was monitored spectrophotometrically at 540 nm, using a standard curve of absorbance at wavelength of 540 nm against different starch concentrations (0–500 mg L^{-1}). One unit of amylase activity is defined as the amount of starch (mg) hydrolyzed during 30 min per mL homogenate at 37 °C.

All assays of digestive enzyme activity were carried out in triplicate using the Bio-Rad Benchmark Plus microplate spectrophotomer and Falcon flat-bottom 96-well microplates (Fisher Scientific). All pH values listed for buffers were measured at room temperature, and all reagents were purchased from Sigma-Aldrich Chemical. Each enzyme activity was measured in each individual fish, and blanks consisting of substrate only and sample only (in buffer) were conducted simultaneously to control for background activity (i.e. activity by endogenous substrate and/or product in the tissue homogenates and substrate solutions). Background was accounted for by substracting the measured OD of the sample with OD of background control prior to plotting on the standard curve. The total protein content was measured according to the method of Bradford (1976) using bovine serum albumin as standard. The specific activity of measured enzymes is expressed as unit enzyme activity per mg protein (U mg $protein^{-1}$).

Total protein and lipid content analyses

At the end of the experiment, remaining fish from each replicate tank were pooled (total wet weight of 2-3 g) and the total protein and lipid content in the whole-fish body of each treatment was analysed following the standard methods. Nitrogen content was analyzed by the Kjeldahl method (AOAC, 2000) and crude protein content was estimated by multiplying nitrogen percentage by 6.25. Lipid content in the whole body was analyzed by extraction following the modification of Folch method demonstrated by Ways and Hanahan (1964). Fatty acid composition was determined by gas chromatography. Fatty acid methyl ester (FAME) analysis was performed following a modified procedure of Lepage and Roy (1984).

Statistical analyses

Normalization of the distribution of the survival and final body weight data were done using arcsin and log transformation, respectively. Comparison of the fish survival, final body weight and length, SGR, FCR, and digestive enzyme activities were done using one-way analysis of variance (ANOVA) analysis. Grouping of treatments based on significant differences in mean values was done according to Tukey test (0.05 level of confidence). STATISTICA statistical software (version 7.0) was used for these statistical analyses.

Results

Fish survival and growth parameters

The initial mean fish body weight was 26.4±2.4 mg which after 28 days of feeding increased to 218±101, 284±61, 269±68, and 258±48 mg for the 0, 5, 25, and 50 g kg^{-1} PHB treatment, respectively (Table 2.2). Each PHB treatment obtained similar SGR value as the control group, with the range of mean SGR of 5.2 to 5.4 % body weight (BW) gain per day (Table 2.2). The FCR expresses the amount of feed dry matter needed per unit of fish gain. No significant difference was observed in the FCR values of the four experimental diet treatments following 4 weeks of feeding, with

the range of mean FCR of 0.8 to 1.0 (Table 2.2). Similarly, no significant difference ($P > 0.05$) was observed among the fish fed with different experimental diets in case of final body length and survival (Table 2.2).

Table 2.2. Survival and growth performance (Mean±SD) of Nile tilapia juvenile fed four experimental diets. No significant differences ($P > 0.05$) were found in the fish survival, final body weight and length, and FCR (n = 3; m = 6). n = number of replicates; m = number of fish pooled per replicate.

Treatment	Survival (%)	Final body weight (mg)	Final body length (cm)	SGR (% BW day^{-1})	FCR
Control	87±2	218±101	2.3±0.4	5.27±0.06^{ab}	1.0±0.3
5 g kg^{-1} PHB	74±8	284±61	2.6±0.3	5.40±0.08^{b}	0.9±0.0
25 g kg^{-1} PHB	86±10	269±68	2.5±0.4	5.25±0.05^{a}	0.9±0.1
50 g kg^{-1} PHB	88±3	258±48	2.5±0.4	5.21±0.03^{a}	0.8±0.1

Different letter within column denote significant differences ($P \leq 0.05$).

Digestive enzymes activity

After 28 days of feeding with the experimental diets, no differences were observed for trypsin, amylase and pepsin activity among the various treatments (Table 2.3) ($P > 0.05$). Feeding the fish with higher levels of PHB significantly increased the activity of lipase in fish fed the 25 g kg^{-1} and 50 g kg^{-1} PHB treatment groups, compared to the control group with the lowest lipase activity (Table 2.3).

Fish body composition

Similar total protein content was observed among all treatments ($P > 0.05$), with the total protein content of 58.2±1.5, 59.4±1.0, 58.6±1.7 and 59.4±2.1 % dry weight (DW) in the control, 5 g kg^{-1}, 25 g kg^{-1} and 50 g kg^{-1} PHB treatment groups, respectively. Dietary PHB supplementation significantly increased the whole-body total lipid content with about 3 % on DW when compared to the control treatment ($P \leq 0.05$) (Table 2.4).

Table 2.3. Digestive enzyme activities (Mean±SD) of Nile tilapia juvenile following 4 weeks of feeding with different experimental PHB-supplemented diets (n = 3; m = 3). n = number of replicates; m = number of fish pooled per replicate.

Treatment	Digestive enzyme activities (U mg protein^{-1})			
	Lipase	Trypsin	Amylase	Pepsin
Control	0.021±0.003^{a}	0.035±0.017	0.457±0.039	0.253±0.010
5 g kg^{-1} PHB	0.025±0.004^{ab}	0.032±0.017	0.533±0.041	0.261±0.021
25 g kg^{-1} PHB	0.029±0.004^{b}	0.033±0.014	0.489±0.042	0.272±0.014
50 g kg^{-1} PHB	0.029±0.001^{b}	0.047±0.016	0.547±0.043	0.245±0.022

Activities are expressed as follows: Lipase as mmole of substrate hydrolyzed min^{-1} mg protein^{-1}; Trypsin activity as hydrolyses mmole of BAPNA min^{-1} mg protein^{-1}; Amylase mg starch hydrolyzed min^{-1} mg protein^{-1}; Pepsin activity as mmole of tyrosine released min^{-1} mg protein^{-1}. Different letter within column denote significant differences ($P \leq 0.05$).

Fish lipid profile

The fish lipids profile (content and composition) following 28 days of feeding on different diets is presented in Table 2.4. In general, the fish fed PHB diets had a higher content of saturated, monounsaturated and (n-6) FAs than the fish fed control diet. The content of saturated and monounsaturated FAs were higher in the 5 g kg^{-1} and 25 g kg^{-1} PHB treatment groups as compared to the control treatment ($P \leq 0.05$), while a higher content of total (n-6) FAs ($P \leq 0.05$) was observed in the 50 g kg^{-1} PHB treatment group. However, PHB did not seem to influence the lipids composition as similar values ($P > 0.05$) were observed among treatments, when the FAs are expressed as percentage of the total lipid content. PHB had no influence over either the content or composition of C20:5n3 (eicosapentaenoic acid, EPA), C22:6n3 (docosahexaenoic acid, DHA), total (n-3) FAs, or the (n-6)/(n-3) ratio ($P > 0.05$).

Table 2.4. Effects of dietary PHB supplementation on the whole-body total lipid content (% DW; Mean±SD) and the main group of long chain fatty acids composition (C14 to C24) as % DW (Mean±SD) and as % total lipid content (in parenthesis; Mean±SD) in Nile tilapia juvenile fed with different PHB experimental diets (n = 3; m = 10). n = number of replicates; m = number of fish pooled per replicate for FAME analysis.

Treatment	Total lipid	FA composition						(n-6)/(n-3) ratio
		Saturated	Mono-Unsaturated	EPA	DHA	Total (n-6)	Total (n-3)	
Control	25.40±0.33^{a}	7.22 ± 0.16^{a} (28.44±0.62)	5.99 ± 0.16^{a} (23.59±0.61)	0.06 ± 0.03 (0.24±0.01)	1.45 ± 0.14 (5.71±0.54)	7.18 ± 0.03^{a} (28.27±0.12)	1.91 ± 0.13 (7.53±0.51)	3.76±0.25
5 g kg^{-1} PHB	28.26±0.13^{b}	8.23 ± 0.35^{bc} (29.13±1.25)	6.68 ± 0.23^{b} (23.64±0.80)	0.07 ± 0.06 (0.26±0.02)	1.65 ± 0.02 (5.83±0.08)	7.61 ± 0.47^{ab} (26.94±1.67)	2.13 ± 0.23 (7.54±0.80)	3.58±0.16
25 g kg^{-1} PHB	27.69±1.06^{b}	8.47 ± 0.17^{c} (30.58±0.60)	6.79 ± 0.25^{b} (24.51±0.89)	0.07 ± 0.08 (0.26±0.03)	1.51 ± 0.13 (5.45±0.48)	7.32 ± 0.41^{ab} (26.44±1.48)	1.95 ± 0.16 (7.03±0.56)	3.76±0.10
50 g kg^{-1} PHB	27.92±0.13^{b}	7.82 ± 0.24^{ab} (27.99±0.86)	6.36 ± 0.06^{ab} (22.78±0.23)	0.07 ± 0.02 (0.27±0.01)	1.63 ± 0.02 (5.82±0.07)	8.09 ± 0.23^{b} (28.95±0.81)	2.10 ± 0.03 (7.52±0.10)	3.85±0.14

Different letter within column denote significant differences ($P \leq 0.05$).

Discussion

Fish growth modulation by dietary PHB supplementation

Although positive effects of PHB on the growth performance in European sea bass juveniles and giant freshwater prawn larvae are reported in the studies by De Schryver et al. (2010) and Nhan et al. (2010), in this study no significant difference was found between treatment groups. Nevertheless, the similar mean final body weights observed in the PHB treatment groups (258 to 284 mg) when compared to the control group (218 mg) indicate that PHB does not have negative effect on the growth of Nile tilapia juvenile. This result corresponds with the study by Najdegerami et al. (2012), where no significant effect of PHB on growth performance of Siberian sturgeon fingerling was observed.

Digestive enzymes activity under dietary PHB supplementation

Digestive enzyme activities can be used as an indicator of digestive processes and fish nutritional conditions (Lazo et al., 2000; Ueberschar, 1988). Various intestinal enzymes involved in digestive and absorptive processes have been reported in tilapia culture, including amylase, pepsin, trypsin, esterases and alkaline phosphatase (Dai et al., 2009; Moriarty, 1973; Tengjaroenkul et al., 2000; Zahran et al., 2014). As for other herbivorous fish, tilapia commonly demonstrate a higher activity of carbohydrase than protease and a lower lipase activity as compared to carnivorous and omnivorous fish (Das and Tripathi, 1991; Opuszynski and Shireman, 1995; Tocher, 2003). However, it has been reported that manipulation of diets causes immediate changes in activities of digestive enzymes (Mohapatra et al., 2012). This specifically holds true for fishes with relatively broad diets (German et al., 2004). In this study, PHB dietary supplementation resulted in significantly higher lipase activities in the 25 and 50 g kg^{-1} PHB-fed fish group, while a similar activity of trypsin, amylase, and pepsin was observed among all different treatments. Lipases are the enzymes which are responsible for the breakdown of dietary lipid complexes within the intestinal lumen (Karasov and Hume, 1997). The

significant increase in lipase activity in the 25 and 50 g kg^{-1} PHB treatment groups (20-40 % higher than in the control group) suggests that the dietary PHB stimulated the dietary lipids digestion, providing more fatty acids available for absorption and esterification into complex lipids. Such increase could be physiologically relevant as a study by Essa et al. (2010) reported a 35-80 % increase in gut lipase activity in Nile tilapia fingerlings (~ 25 g) due to supplementation of probiotic *Bacillus subtilis* and *Lactobacillus plantarum* (either as single or mixture inoculum), which also resulted in better growth and feed utilization obtained in the treatment group. Further investigation is needed to confirm the relevance of lipase activity in feed utilization (i.e. lipids digestion) by PHB supplementation, e.g. by the use of exogenous lipase along with PHB supplementation.

Dietary PHB supplementation did not affect lipid profile of Nile tilapia juvenile

It is reported by Najdegerami et al. (2012) that crystalline PHB significantly increased the linoleic acid and total (n-6) FAs content in the liver of Siberian sturgeon fingerling fed on 5 g kg^{-1} PHB diet. In another study, crystalline PHB was found to increase the total monounsaturated and total (n-3) FAs content of giant tiger prawn postlarvae (Ludevese-Pascual, personal communication). Similarly, in this study, an increasing trend was observed in the total saturated, total monounsaturated FAs and total (n-6) FAs content (% DW) of Nile tilapia juvenile. However, PHB did not affect the composition of FAs groups, be it the total saturated FAs, monounsaturated FAs, (n-3) FAs (including EPA and DHA), or (n-6) FAs, when they are expressed as % total lipid content. The FAME analysis procedure performed in this study only measured the long chain fatty acids (LCFAs; C14 to C24) and did not provide information on the composition of short chain fatty acids (SCFAs; C2 to C6) or medium chain fatty acids (MCFAs; C8 to C12). As the degradation of dietary PHB is hypothesized to results in the release of SCFA, likely β-HB (De Schryver et al., 2010; Defoirdt et al., 2009), it might be interesting to evaluate the effect of PHB on the fish SCFAs profile.

Fish total lipid content increased with dietary PHB supplementation

Although there was no effect of PHB on the FAs profile, PHB was found to increase the fish whole-body total lipid content. It was mentioned earlier that the increasing trends in lipase activity by the PHB treatment groups might indicate that dietary PHB can stimulate lipids digestion, and thus may have been responsible in the significant increase in fish total lipid content in the PHB treatment groups when compared to the control group. Lipids can be divided into two main classes, i.e. polar lipids (PLs) and neutral lipids (NLs). PLs are important constituents of membranes and they function as precursors in the eicosanoid metabolism (structural / functional fat), while the NLs serve mainly as a depot of lipids used as an energy source (depot / storage fat) (Henderson and Tocher, 1987). The contribution of PLs and NLs to the total lipid was not determined in this study; however, Suloma et al. (2008) suggested that PLs make up about 80 % of the total lipid in tilapia.

Semova et al. (2012) suggested that altered fish fat metabolism and lipid profiles could be due to the effects of GI microbiota on: (i) intestinal absorption or metabolism of exogenous dietary lipids, or (2) hepatic production or metabolism of endogenous lipids. In this study, it can only be hypothesized that the increase of fish total lipid content may have resulted from the diet (PHB)-induced alterations in the intestinal microbiota composition. Further studies on the microbiota-diet interactions are necessary to investigate any potential impact of the PHB on the intestinal microbiota composition and its importance in the regulation of dietary nutrients (i.e. lipids) digestion and metabolism.

The observed total lipid content can be favourable for human consumption, as lipids play an essential role as energy and FAs source for human, both for immediate utilization by the body and in laying down a storage depot (adipose tissue) for later utilization when food intake is reduced, and also act as a vehicle for the ingestion absorption of fat-soluble vitamins (Medeiros and Wildman, 2013). Structural fats are specifically of interest as they are needed inside every cell in

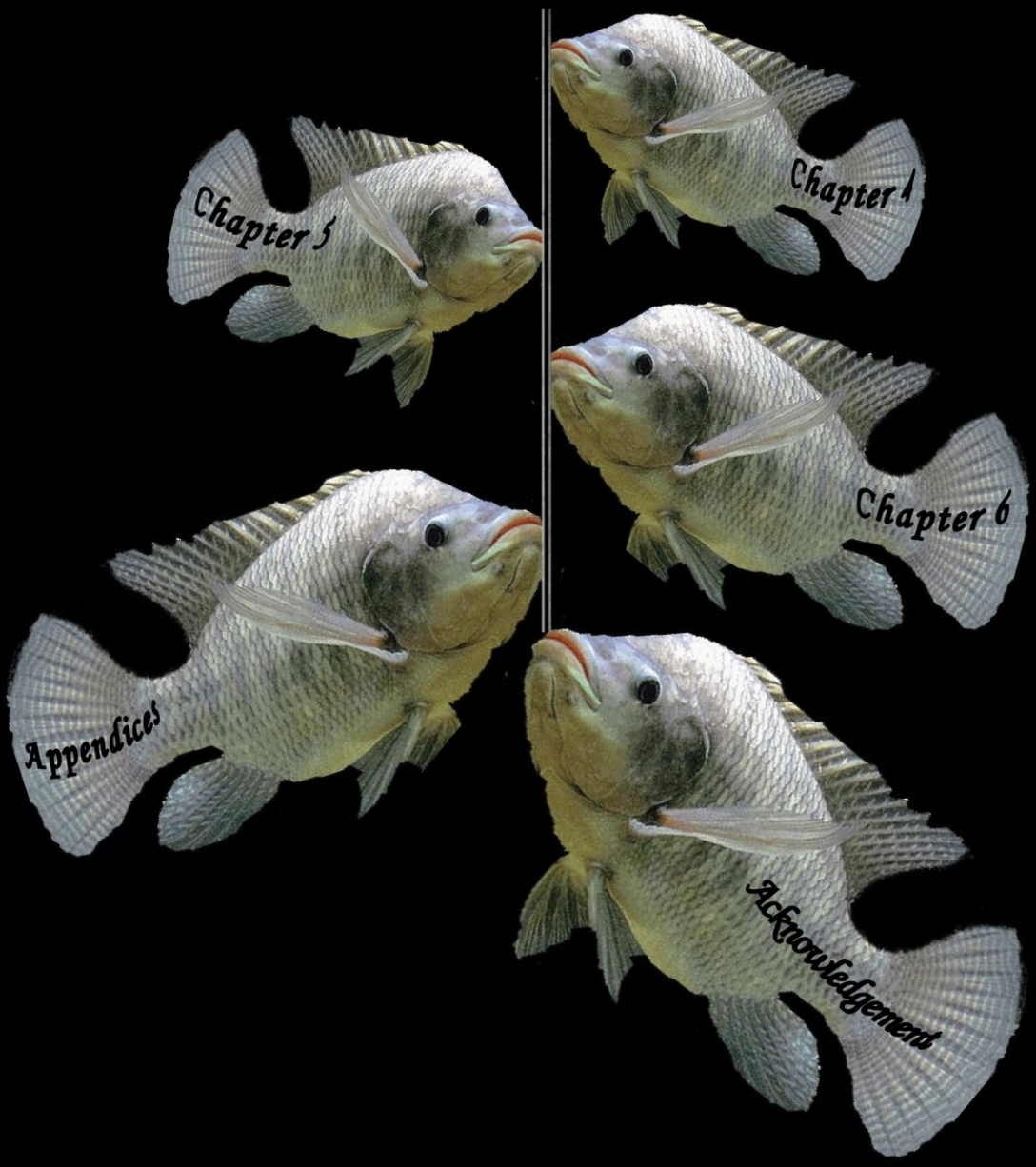
human bodies and are especially necessary for the growth of the fetal brain (FAO, 2010; Uauy et al., 2000). It should thus be verified if the increment of total lipid resulting from PHB supplementation led to a higher eicosanoid metabolism which may have resulted in more structural fat production, or that it was more neutral fat that was accumulated which does not contribute to the quality of the tilapia for consumption.

Conclusions

In general, the findings of this study suggest that PHB do not have negative effect on the growth of Nile tilapia juvenile. Eventhough a trend of increasing growth was observed in all PHB treatment groups, the increase was not statistically significant compared to the control group. Furthermore, lipid digestion and deposition seemed to increase by the dietary PHB supplementation. We hypothesize that the altered lipid utilization may have resulted from diet-induced alterations in the GI microbiota composition. Further research needs to focus on the effect of PHB on the modulation of the digestive functionality of the GI MC. Studies on the host microbiota-diet interactions (i.e. by making use of physiological techniques such as Biolog microplate analysis) are expected to contribute to a more advanced knowledge of the possible mechanisms of action of PHB in the alteration of nutrient utilization by the host, which could potentially result in growth enhancement in aquaculture practices.

Chapter 3

Use of community level physiological profiling of the gut microbiota in Nile tilapia fingerlings to investigate the growth promoting effect of **poly-β-hydroxybutyrate**



Chapter 3

Use of community level physiological profiling of the gut microbiota in Nile tilapia fingerlings to investigate the growth promoting effect of poly-β-hydroxybutyrate

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[a] *Laboratory of Aquaculture & Artemia Reference Center (ARC), Ghent University, Belgium*

[b] *School of Life Sciences and Technology, Bandung Institute of Technology, Indonesia*

[c] *College of Veterinary Medicine, Mekelle University, Mekelle, Ethiopia*

Abstract

The bacterial storage polymer poly-β-hydroxybutyrate (PHB) has a high potential as a feed additive to promote the growth and health of fish and shrimp. In the current study, the effect of crystalline PHB on the growth performance and survival of Nile tilapia (*Oreochromis niloticus*) fingerlings was evaluated. The tilapia fingerlings were cultured for 4 weeks with diets containing 6 different PHB levels: 0 (control), 1, 2.5, 5, 25 and 50 g PHB kg^{-1} diet. The control and PHB treatment groups showed no significant differences in survival while the growth performance of the fingerlings was significantly higher when PHB was present in the diet. No significant differences in growth were observed between the different PHB treatments. The digestive functionality of the gut microbial communities was evaluated using the Biolog EcoPlate™ method. This allowed to successfully discriminate between the indigenous (mucosa associated) and transient (faeces associated) gastrointestinal microbial community (MC). The former showed a lower functional diversity and functional evenness but a higher utilization intensity as compared to the latter. Based on the 31 substrates included in the Biolog EcoPlate™ method, no significant effect of PHB on the functional profiling of the gut communities could be detected. Based on the results, it is suggested that changes in tilapia fingerlings growth performance resulting from PHB supplementation is not necessarily related to a change in the gut microbial digestion patterns. Therefore, further studies are needed to make the link between PHB supplementation and its growth enhancing effect.

Introduction

The disease controlling effects of PHB have been shown in a number of studies. Biodegradation of PHB by microbial enzymes and/or host digestive enzymes, is hypothesized to yield β-hydroxybutyrate which is assumed to induce an antimicrobial effect (Defoirdt et al., 2007a). The supplementation of PHB, both in crystalline and amorphous forms, resulted in a significant protection of *Artemia* nauplii (Defoirdt et al., 2007b), giant freshwater prawn (Thai et al., 2014) and giant tiger prawn larvae (Laranja et al., 2014) against vibriosis. Equally interesting, the dietary supplementation of PHB to commercial feed resulted in improved growth performance and/or survival of European sea bass juveniles (De Schryver et al., 2010), Siberian sturgeon fingerlings (Najdegerami et al., 2011), Chinese mitten crab larvae (Sui et al., 2012), giant freshwater prawn larvae (Nhan et al., 2010; Thai et al., 2014) and giant tiger prawn larvae (Laranja et al., 2014). An explanation for the growth promoting effect of PHB is, however, so far lacking.

It was highlighted by Tanaka et al. (2004), Ringø et al. (2006), and Yang et al. (2007), that the type of food is important for the composition and activity of a fish gastrointestinal (GI) microbiota. This microbiota is classified as 'autochthonous' or 'indigenous' when it is associated with the host's gut epithelial surface or as 'allochthonous' or 'transient' when it is mainly residing in the lumen and associated with the faeces (Denev et al., 2009). It has been shown that dietary PHB supplementation modulates the fish gut MC organization in terms of genetic diversity, relative abundances, and composition dynamics (De Schryver et al., 2010). The increased diversity and evenness of the gut microbiota may be a valuable contributor to an increased resistance against pathogenic infections (De Schryver et al., 2010). In terms of nutrition, many studies indicate that fish gut microbiota contribute by an important digestive and nutritional function to the intestinal metabolism and absorption of ingested food (Cummings and Englyst, 1995; Ganguly and Prasad, 2012; Macfarlane and Macfarlane, 2007; Mouchet et al., 2012; Semova et al., 2012; Stevens and Hume, 1998). More specifically, several

studies reported the preponderance of aerobic, microaerophilic and facultative anaerobic bacteria in fish digestive process (Ganguly and Prasad, 2012; Nayak, 2010; Saha et al., 2006). Modulation of the intestinal microbiota may thus also result in a modification of gut metabolic activity and feed digestion. Also, several studies suggested that certain feed ingredients and/or feeding intensity may affect the qualitative and quantitative composition of intestinal microbiota of fish (Pucci et al., 2000; Ringo and Olsen, 1999; Skrodenyte-Arbaeiauskiene, 2000; Voverienė et al., 2002) and thus might change the digestive pattern by the microbiota in the gut.

In addition to other important factors, including culture systems, water, and diet, fish age / developmental stage also influences the gut microbiota composition. Tengjaroenkul et al. (2000) reported that the gross intestinal configuration of Nile tilapia intestine changes dramatically from a short, straight intestinal tube at hatch (day 0) to a very complex, coiling pattern first attained at age 9 weeks old, until it reaches the complexly-coiled definitive form as found in adult fish at age 12 weeks old. It is suggested that at younger age (< 12 weeks old) tilapia is considered to have rather simpler gut that would favour colonization of aerobic, microaerophilic or facultative anaerobs bacteria, while the adults have complexly-coiled definitive form that would favour more colonization of anaerobic bacteria (Ferguson et al., 2010). Several studies reported that microaerophilic and facultative anaerobic probionts, including *Bacillus* sp., and *Lactobacillus* sp., have been applied to promote growth and health of Nile tilapia fingerlings (Aly et al., 2008; Mohamed and Refat, 2011; Pirarat et al., 2006; Zhou et al., 2010).

The aim of the current study was therefore to investigate how supplementation of PHB to the diet of Nile tilapia fingerlings changed the functional activity of the aerobic / microaerophilic gut MC and if this could be related to the culture performance of the fish. Consequently, the effect of different dietary levels of PHB (0, 1, 2.5, 5, 25, and 50 g kg^{-1} diet) on (i) the fish growth and survival performances, and (ii) the functional physiological diversity of the indigenous (intestine samples)

and the transient (faeces samples) gut MC of Nile tilapia fingerlings using Biolog EcoPlate™ method was evaluated.

Materials and Methods

Experimental fish and husbandry

A total of 360 Nile tilapia fingerlings (~ 0.6 g) produced by Til-Aqua International (Velden, The Netherlands) were used in the experiment. Upon arrival in the lab, the fish were housed for one week in a 500 L cylindrical tank in the experimental chamber to acclimatize to the temperature, light regime and experimental semi-purified diet (control diet without PHB). The fish were fed three times daily to apparent satiation. At the end of the acclimation period, the fish (average body weight of 0.8±0.1 g) were randomly distributed over 24 tanks of 40 L (6 treatments, 4 replicates) at a density of 15 fish per tank. During the experiment, a daily water renewal of ~30 % of the volume in the tanks was done to maintain NO_2^- and NH_4^+ below 1 mg NO_2^--N L^{-1} and 1 mg NH_4^+-N L^{-1}, respectively. The culture water was continuously aerated with air stones ensuring dissolved oxygen above 5 mg O_2 L^{-1}, and the photoperiod was maintained on a 16h:8h light:dark schedule. During the 4 weeks feeding experiment, water temperature was maintained at 27±0.5 °C using electrical heaters.

Feed Formulation and Feeding Design

A basal semi-purified diet (control diet) was formulated as described in Chapter 2 (Table 2.1). For the PHB experimental diets, crystalline PHB (± 98 % purity, average 300 μm size; Goodfellow Cambridge Limited, Huntingdon, England) was supplemented to the basal diet at five levels – 1, 2.5, 5, 25, and 50 g PHB kg^{-1} diet – at the expense of α-cellulose. The dry ingredients of each diet were thoroughly mixed in a Hobart mixer (Hobart Corp., Troy, Idaho, USA) before liquid and oily ingredients were added. The moist mixture was extruded through a 3 mm diameter grinder plate in a Hobart meat grinder. The resulting moist pellets were

air-dried at room temperature to a moisture content of about 100 g kg^{-1}. Pellets were ground into small pieces and sieved to obtain a size of approximately 800 μm and stored frozen in plastic bags at −20 °C until fed. The fish were fed at a fixed daily feeding level of 6 % on body weight (Ng and Romano, 2013) in three equal meals given every 4 hours between 09:00 and 17:00 during the 4 weeks experimental period. On a weekly basis the daily feeding quantity for each tank was adjusted based on the fish wet weight measured every week. All experimental diets were evaluated in 4 replicates.

Growth and survival parameters

Every week, five fish from each replicate tank were randomly collected and weighted after a 24 h starvation period to monitor the growth performance. The average weight gain of the fish in a tank over the 4 weeks was calculated by subtracting the weight of the fish sampled on the final day of the experiment with the average weight of the fish as measured at the beginning of the experiment, and then taking the average. The specific growth rate (SGR) was calculated as described in Chapter 2:

$$\text{SGR (\% body weight gain per day)} = \left[\frac{(\ln W - \ln W_0)}{t}\right]$$

where W is the average weight after 4 weeks, W_0 is the average initial weight (measured at the beginning of the experiment), and t is experiment period (4 weeks). The same approach was used to calculate the feed conversion ratio (FCR), expressed as the feed consumption over the weight increase of the fish per treatment. Fish survival of each treatment group was determined at the end of the experimental period by computing the number of surviving fish relative to the number of fish at the beginning of the experimental period.

Proximate analysis

After 4 weeks of feeding with the different foods, the protein and lipid content of the whole body of 5 fish per tank was analyzed following the standard methods.

Nitrogen content was analyzed by the Kjeldahl method (AOAC, 2000) and crude protein content was estimated by multiplying the nitrogen percentage with 6.25. The lipid content in the whole body was analyzed by extraction following the Folch method illustrated by Ways and Hanahan (1964) in which the fatty acid composition was determined by gas chromatography. Fatty acid methyl ester (FAME) was prepared following a modified procedure of Lepage and Roy (1984).

Community-level physiological profiling (CLPP)

Sample collection

Fish were sampled for the analysis of the functional diversity of the indigenous and transient bacterial communities at the end of the experimental period. From each tank, 5 fish were randomly sampled and transferred into a single 4 L tank containing clean fresh water for a 24 h starvation period. Then, faecal samples were collected from each tank making up a pooled faecal sample for this respective tank. Next, the sampled fish were euthanized in an ice-cold 5 g L^{-1} benzocaine solution (Sigma, E-1501) and aseptically dissected for removal of the whole gut. The collected intestine was gently squeezed to remove any remaining contents and then rinsed gently with 9 g L^{-1} NaCl sterile saline solution. The intestine tissues from the fish from one tank were pooled.

Analysis of the sampled microbiota – the Biolog EcoPlate assay

Pooled samples were homogenised with sterile 0.5 mm glass beads in sterile screw-capped tubes using a bead beater (4 x 15 s, each with 15 s break). Sterile saline solution (9 g L^{-1} NaCl) was used as a homogenization solvent at a ratio of 25 μL for every mg of sample. Homogenized samples were then put in a stand for 30 minutes after which the supernatant was collected carefully not to include any particulate matter, and diluted 100 times. The diluted supernatant was then filtered using a 100 μm cell strainer (SPL Cell Strainer, SPL Life Sciences) and the filtrate was applied in the Biolog assay. Due to higher mortality recorded in some

treatment replicates, only three out of four replicates of each treatment were analysed in the Biolog assay.

The Biolog EcoPlate™ (BIOLOG Inc., Hayward, Calif.) assay was used for characterizing community-level physiological profiles of the faecal and gut microbial communities. The wells on the 96-well plates contain carbon sources and a tetrazolium violet redox dye that turns purple if inoculated microorganisms utilize these sources. The Biolog EcoPlate™ contains 31 different carbon sources, allowing for community-level physiological profiling (CLPP) of heterotrophic bacterial assemblages. The Biolog EcoPlate™ that was used in this study has three replicates of a set consisting of 31 substrates and one blank well without substrate. Each filtrate sample was inoculated into one Biolog EcoPlate™ (150 μL of sample homogenate per well) and initial absorbance (0 h) at 590 nm (OD_{590}, absorbance peak of tetrazolium) was measured as a reference using a TECAN Infinite 200 spectrophotometer (Tecan, Switserland). All Biolog EcoPlates were incubated aerobically at 28 °C in a dark chamber and the absorbance values of colour development in the wells were recorded after 24, 48 and 84 h of incubation.

Data Management and Analysis

a. Biolog Plate assay data

The raw absorbance values obtained from spectrophotometric readings were first blanked against their control wells in each replicate part of the individual plates and negative values were rounded to zero (Kamitani et al., 2006; Van Beelen et al., 2004).

b. Average Well Colour Development

To normalize for variations in the amount of bacterial biomass loaded in the wells among replicate samples, the average well colour development (AWCD) for replicate parts in individual plates was determined. The AWCD was calculated per reading time as follows:

$$AWCD = \frac{\sum(C - R)}{n}$$

, where: C is the OD of an individual well, R is the OD of the control well and n is the total number of wells in each part of the EcoPlate (n = 31). Normalization of OD data was then carried out by dividing each blank-corrected well by the AWCD of that plate, *i.e.* (C–R)/AWCD (Garland and Mills, 1994; Garland, 1996). Statistical multivariate comparisons were then carried out on the normalized data.

c. Diversity of substrate utilisation (DSU)

DSU was determined by the Shannon-Wiener index (H), which is one of the most commonly used diversity indices considering both the number of species and their abundance (Muñiz et al., 2014; Tuomisto, 2010). In this case, the H value considers the number of used carbon sources and their utilization intensity thus describing the ability of the bacterial community to degrade carbon sources resulting in an index of physiological diversity of the bacterial community. Microbial communities that are able to degrade more substrates and/or to degrade them with a higher efficiency would have higher values of H. The index was calculated as follows (Tam et al., 2001; Wang et al., 2011):

$$H = -\sum p_i \, (\ln p_i)$$

, where p_i is the normalised value of OD_{590}.

d. Gini coefficient

For calculation of the Gini coefficient, the normalized OD_{590} values from individual wells from a homogenate sample were ranked from low to high and the cumulative proportion of absorbance (from 0 to 1) was plotted against the cumulative normalized number of the wells (from 0 to 1). This can be depicted as the Lorenz curve. Perfect equality, achieved only by completely even absorbance in each well would result in a diagonal line from the origin to the upper right corner. The Gini coefficient represents the degree of deviation from this diagonal, and it is evaluated

as twice the area between the diagonal and the Lorenz curve (Ogwang and Rao, 1996) using the following equation:

$$G = 1 - \sum_{k=1}^{n} (X_k - X_{k-1})(Y_k + Y_{k-1})$$

, where X_k is the cumulative normalized number of the wells, with $X_0 = 0$, $X_n = 1$; and Y_k is the cumulative proportion of absorbance, with $Y_0 = 0$, $Y_n = 1$. n is the total number of carbon sources (31). The Gini coefficient has a minimum value of 0, when all wells have equal absorbance and a theoretical maximum of 1.0, in which all wells but one have a value of 0.0.

e. Principal component analysis (PCA)

PCA was performed to reduce the complex nature of the data (31 variables per sample) into smaller and interpretable representative variables in an ordination space (Garland and Mills, 1991; Garland and Mills, 1994). Relationships among treatments were obtained by plotting the correlation scores of their first two PCs in two dimensions.

Statistical Analyses

Comparison of the fish survival, BW gain, SGR, and FCR were done using one-way analysis of variance (one-way ANOVA). For fish survival, arcsine transformation of survival data was used. Correlation analysis (Spearman Rank Order Correlations test) between fish survival and weight gain was conducted to investigate whether any relationship existed between the parameters. Grouping of treatments based on significant differences in mean values was done using a Tukey post-hoc test (0.05 level of confidence).

For the community level physiological profiling, PCA was performed on the normalised absorbance data at 84 h. Substrate richness was calculated as the number of highly utilized substrates (corrected absorbance > AWCD) and the percentage of compounds used in each of the substrate classes: polymers (4

compounds), carbohydrates (10 compounds), carboxylic acids (7 compounds), amino acids (6 compounds), amines (2 compounds) and phenolic (2 compounds). For each of the sample types, substrate richness indices and percentages of utilization (arcsin square root transformed data) were analysed using one-way ANOVA.

A two-way analysis of covariance (two-way ANCOVA; factors of different PHB treatment and incubation time of Biolog EcoPlate) was conducted for the Shannon-Wiener index and the Gini coefficient of each sample type (intestine and faeces), using AWCD as a covariate to remove the effect of differential rates of colour development across the plates (Harch et al., 1997). All statistical analyses were completed using STATISTICA statistical software (version 7.0).

Results

Fish performance

The effects of the experimental treatments were examined with respect to body weight (BW) gain, specific growth rate (SGR), and survival of the fingerling. After 4 weeks, no significant differences in survival could be observed ($P > 0.05$; Table 3.1). All PHB treatments showed a significantly higher mean body weight gain and specific growth rate and a significantly lower FCR than the control group ($P \leq 0.05$), while no significant differences were detected for these parameters between the different PHB treatments (Table 3.1).

The BW gain after 4 weeks of feeding was equal to 177±10 %, 265±59 %, 296±64 %, 303±94 %, 296±16 %, and 312±48 % of the initial body weight in the control, 1 g kg^{-1}, 2.5 g kg^{-1}, 5 g kg^{-1}, 25 g kg^{-1} and 50 g kg^{-1} PHB treatment, respectively. All PHB treatment groups showed a significantly lower FCR as compared to the control group ($P \leq 0.05$). A correlation analysis was performed to assess if fish survival and weight gain were related to each other. There was no significant correlation

between the fish survival and the mean body weight gain in the 6 treatments (Spearman Rank Order Correlations test; r^2= -0.14, $P > 0.05$).

Table 3.1. Survival and growth performance parameters (Mean±SD) of Nile tilapia fingerlings fed with different PHB diets for 4 weeks. Different letters within the same column denote significant differences ($P \leq 0.05$) (n = 4; m = 3). n = number of replicates; m = number of fish pooled per replicate.

Treatment	Survival (%)	BW gain (g)	SGR* (% BW day^{-1})	FCR**
Control	71±19	1.42±0.08[a]	3.64±0.13[a]	1.51±0.17[a]
1 g kg^{-1} PHB	68±27	2.12±0.47[b]	4.59±0.56[b]	1.07±0.21[b]
2.5 g kg^{-1} PHB	93±5	2.37±0.51[b]	4.87±0.60[b]	0.92±0.22[b]
5 g kg^{-1} PHB	46±31	2.43±0.75[b]	4.92±0.78[b]	1.01±0.12[b]
25 g kg^{-1} PHB	60±34	2.37±0.13[b]	4.91±0.15[b]	0.94±0.04[b]
50 g kg^{-1} PHB	78±8	2.50±0.38[b]	5.04±0.42[b]	0.89±0.17[b]

* SGR = specific growth rate. ** FCR = feed conversion ratio

Fish whole body protein and lipid content

No effect of PHB on the crude protein level or the whole-body lipid content in the fingerlings was observed among various treatments ($P > 0.05$; Fig. 3.1). The fatty acid composition of the fingerlings following the 4-week feeding period is presented in Table 3.2. Although all values for the main groups of fatty acids were higher for PHB treatments than for the control treatment, no significant differences among the treatments were observed ($P > 0.05$).

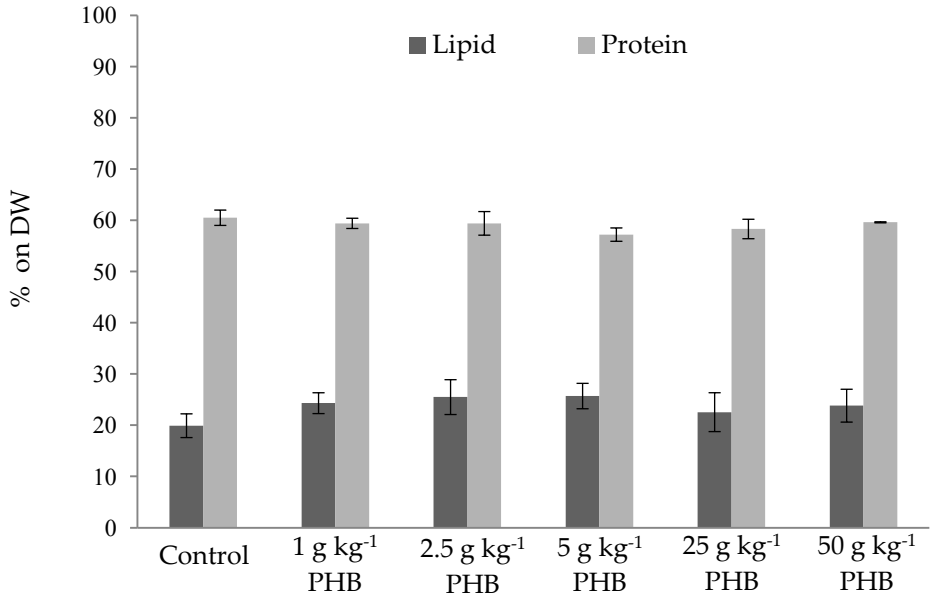


Figure 3.1. Total crude protein and lipid content of whole-body Nile tilapia fingerlings following 4 weeks of feeding with different PHB diets. No significant differences in either total crude protein or lipid content were observed among treatments ($P > 0.05$) (n = 3; m = 5). n = number of replicates; m = number of fish pooled per replicate for analysis.

Table 3.2. Dietary PHB supplementation had no influence on the content of main group of long chain fatty acids (C14 to C24) (% DW; Mean±SD) and their composition as % total lipid content (in parenthesis; Mean±SD) in Nile tilapia fingerlings fed with different PHB diets ($P > 0.05$) (n = 4; m = 5). n = number of replicates; m = number of fish pooled per replicate for FAME analysis.

	Saturated	**Mono-unsaturated**	**DHA**	**EPA**	**Total (n-6)**	**Total (n-3)**
Control	4.63±0.60 (23.22±0.62)	5.68±0.75 (28.49±0.67)	1.54±0.11 (7.77±0.66)	0.30±0.03 (1.51±0.22)	5.85±0.67 (29.4±0.71)	2.38±0.16 (12.00±0.98)
1 g kg^{-1} PHB	5.64±0.48 (23.20±0.16)	7.20±0.70 (29.60±0.60)	1.67±0.08 (6.90±0.37)	0.37±0.06 (1.51±0.16)	7.27±0.57 (29.9±0.71)	2.71±0.22 (11.14±0.38)
2.5 g kg^{-1} PHB	5.62±0.30 (22.30±3.10)	6.77±0.33 (26.87±3.83)	1.60±0.05 (6.37±0.99)	0.31±0.01 (1.24±0.21)	7.00±0.23 (27.8±4.05)	2.49±0.08 (9.91±1.56)
5 g kg^{-1} PHB	5.69±0.57 (22.64±0.12)	6.74±0.82 (29.62±0.21)	1.61±0.13 (7.31±0.51)	0.35±0.04 (1.67±0.10)	7.00±0.71 (29.5±0.02)	2.47±0.22 (12.02±0.41)
25 g kg^{-1} PHB	5.21±0.80 (23.16±0.44)	6.71±1.36 (29.61±1.24)	1.63±0.21 (7.29±0.57)	0.37±0.05 (1.63±0.12)	6.62±1.10 (29.4±0.25)	2.61±0.33 (11.67±0.90)
50 g kg^{-1} PHB	5.35±0.68 (22.50±0.69)	6.66±0.95 (27.97±0.82)	1.67±0.18 (7.03±0.35)	0.34±0.06 (1.43±0.07)	6.88±0.96 (28.9±0.18)	2.61±0.31 (10.97±0.43)

Community-level physiological profiling (CLPP)

AWCD, Shannon-Wiener Index and Gini coefficient

Two-ways ANCOVA was performed on the Shannon-Wiener index (H) and the Gini coefficient (G) data to evaluate the incubation time effect (24, 48, 84 h) and PHB treatment effect (control, 1, 2.5, 5, 25, and 50 g PHB kg^{-1} diet) in the intestine and faeces samples, separately. In general, a lower Gini coefficient and higher Shannon-index were observed in the intestine samples. However, no significant difference was found among the various treatments for all parameters (Table 3.3).

Principal Component Analysis (PCA)

PCA was used to separate the microbial samples based on their utilization of all 31 different carbon sources. In general, PCA of both intestine and faeces samples did not reveal any distinctive patterns between the treatment groups (Fig. 3.2). For both sample types, different PHB treatments are close to each other on the sphere and their projections are very close to the correlation circle thus it can be ascertained that the variables / different treatment groups are highly correlated, i. e., similar to each other. The first two principal components (PCs) accounted for 92.7 % and 3.6 %, respectively, of the total data variation for the intestine samples and 83.5 % and 8.6 %, respectively, of the total data variation for the faeces sample. In the intestine samples, variability in the first PC was explained by a contrast among utilization of amine, phenolic compound, carboxylic acids, while in the faecal samples, variability in the first PC was explained by a contrast among utilization of amine, carboxylic acids, phenolid compound and mixed utilization (both high and low) of amino acids and carbohydrates (Tabel 3.4). Both PCAs could not separate the various treatment groups due to similar levels of utilization of carbon sources.

Table 3.3. Results for the incubation time effect (24, 48, 84 h) and PHB treatment effect (control, 1 g kg^{-1}, 2.5 g kg^{-1}, 5 g kg^{-1}, 25 g kg^{-1}, and 50 g kg^{-1} PHB diet) from two-way ANCOVA of the Shannon-Wiener index (H) and the Gini coefficient (G) for both intestine and faeces samples. No significant difference ($P > 0.05$) was found among different incubation times or various treatments for both parameters (n = 3, m = 5). n = number of replicates; m = number of fish sampled per replicate for analysis.

Variables for analysis	**Incubation time**			**Treatment**					
	24 h	48 h	84 h	Control	1 g kg^{-1} PHB	2.5 g kg^{-1} PHB	5 g kg^{-1} PHB	25 g kg^{-1} PHB	50 g kg^{-1} PHB
Intestine									
Shannon-Wiener index (H)	3.025	3.157	3.198	3.096	3.151	3.113	3.167	3.090	3.144
Gini coefficient (G)	0.479	0.361	0.335	0.414	0.374	0.392	0.367	0.416	0.385
Faeces									
Shannon-Wiener index (H)	3.164	3.325	3.380	3.275	3.278	3.305	3.273	3.305	3.294
Gini coefficient (G)	0.390	0.240	0.165	0.278	0.284	0.254	0.287	0.246	0.255

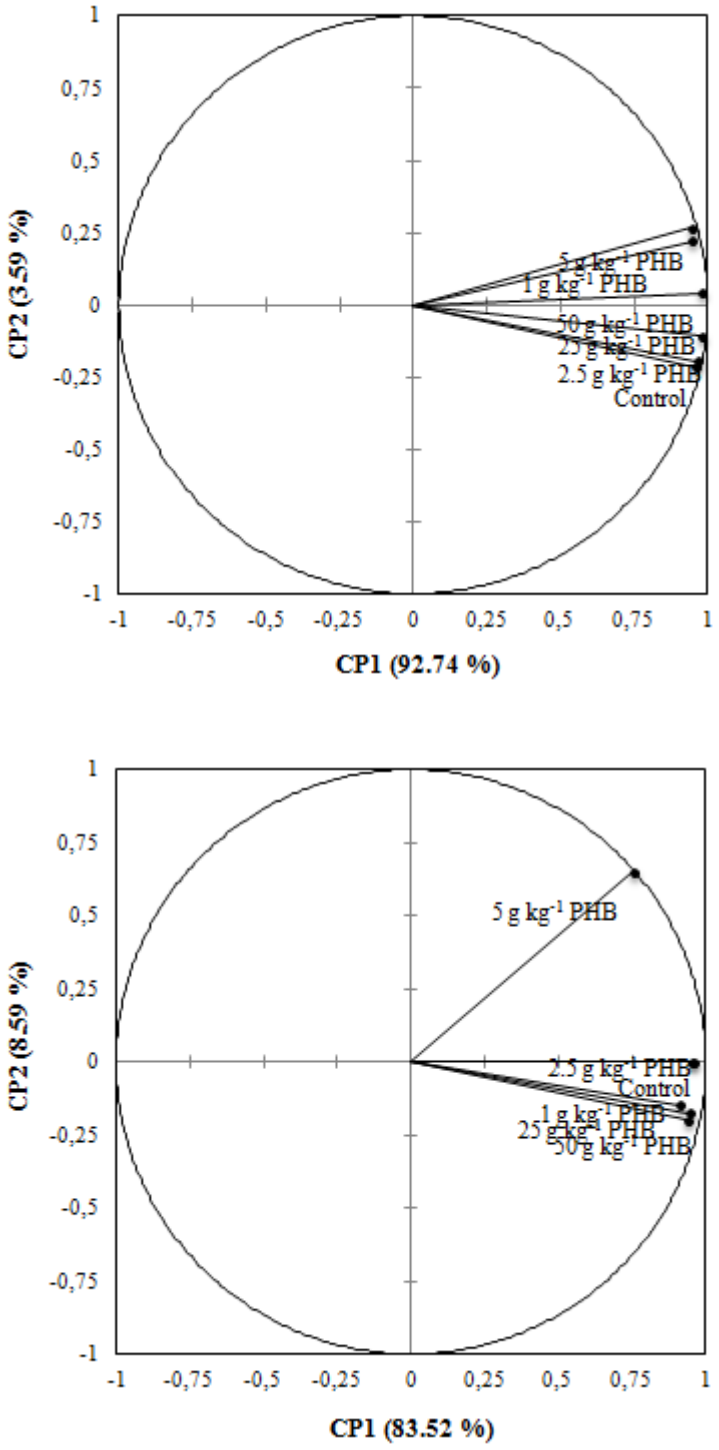


Figure 3.2. Principle component analysis of Biolog assay data for the gut microbiota of tilapia fed with different PHB experimental diets for 4 weeks (n = 3, m = 5). n = number of replicates; m = number of fish sampled per replicate for analysis. The correlation circles for the intestine samples (upper graph) and faeces samples (lower graph) are shown.

Table 3.4. Carbon sources most heavily loaded on first two principal components (PC) in analysis of gut MC characteristics from intestine or faeces sample of 6 dietary PHB treatments. When no value is given, the loading was <0.15 and >-0.15. Carbon sources with asterisk (*) indicate most heavily loaded carbon sources observed in both intestine and faeces sample analysis.

Intestine sample		
	PC1	PC2
Amine		
Phenylethylamine*	0.27	0.16
Amino Acids		
L-Arginin	0.20	-0.17
L-Asparagine*	0.18	
L-Phenylalanine*	-0.20	-0.19
Carbohydrates		
α-D-Lactose*	-0.17	0.45
D-Mannitol	0.19	
i-Erythritol*	-0.35	
Glucose-1-Phosphate*		0.42
D-Xylose*	0.17	0.36
D-Galactonic Acid γ-Lactone	-0.29	-0.30
N-Acetyl-D-Glucosamine	0.16	
D,L-α-Glycerol Phosphate*	-0.22	
Carboxylic Acids		
D-Glucosaminic Acid*	-0.35	
Itaconic Acid*		0.18
γ-Hydroxybutyric Acid*	-0.15	
Polymers		
Tween 40	0.21	
Tween 80	0.18	
α-Cyclodextrin	-0.27	-0.27
Glycogen	0.22	
Phenolic compound		
2-Hydroxy Benzoic Acid*		0.29
4-Hydroxy Benzoic Acid*	0.20	

Faeces sample		
	PC1	PC2
Amine		
Phenylethylamine*	0.33	-0.18
Amino Acids		
L-Asparagine*	0.15	
L-Phenylalanine*	-0.25	-0.22
L-Threonine	-0.23	-0.25
Carbohydrates		
α-D-Lactose*	0.16	
i-Erythritol*	-0.31	0.42
Glucose-1-Phosphate*	-0.15	-0.16
D-Xylose*	0.31	
D,L-α-Glycerol Phosphate*	-0.46	
Carboxylic Acids		
α-Ketobutyric Acid	-0.22	
D-Glucosaminic Acid*	-0.25	0.39
D-Malic Acid		-0.36
Itaconic Acid*		0.37
γ-Hydroxybutyric Acid*	-0.24	
Phenolic compound		
2-Hydroxy Benzoic Acid*	0.19	
4-Hydroxy Benzoic Acid*	0.20	

Substrate richness

Similar values of the mean number of intensively used carbon sources (blanked absorbance > AWCD) were shown by the samples from all treatments (Fig. 3.3). The mean percentage of compounds used in each of the substrate classes (amines, amino acids, carbohydrates, carboxylic acids, polymers and phenolic compounds) for both intestine and faeces samples are shown in Fig. 3.4. Similar utilization of about 60 %, 60 %, 50 %, 40 %, 80 % and 80 % of amine, amino acid, carbohydrate, carboxylic acid, polymers, and phenolic compound, respectively, was observed in the intestine sample of all treatment groups; while about 60 %, 50 %, 60 %, 40 %, 90 % and 100 % utilization of amine, amino acid, carbohydrate, carboxylic acid, polymers, and phenolic compound, respectively, was observed in the faeces sample of all treatment groups. Both intestine and faeces samples did not show any significant difference in either the mean number of carbon sources utilized or the mean percentages of compounds used in each of the substrate classes between treatment groups.

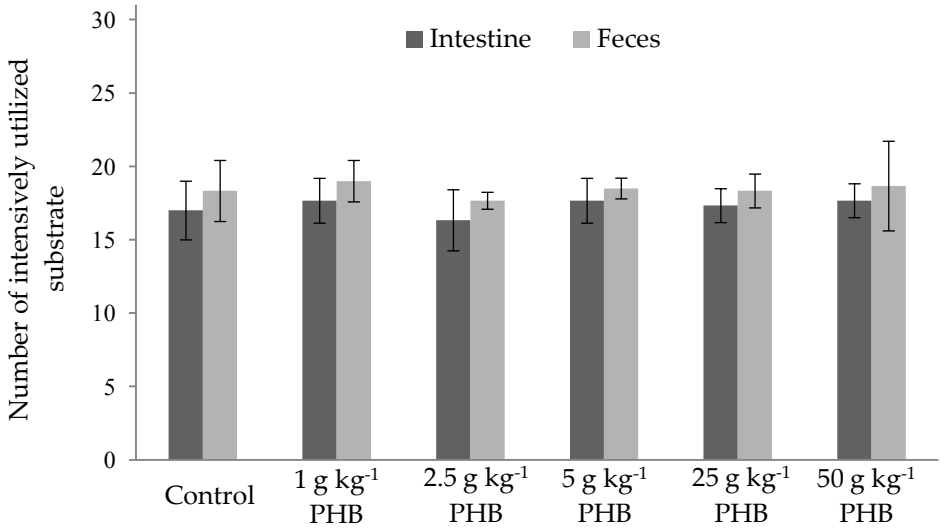


Figure 3.3. Substrate richness: number of intensively used substrates (blanked absorbance > AWCD) of both intestine and faeces samples from the different PHB treatment groups. The bars represent the mean values and error bars represent standard deviation. No significant difference in the number of intensively utilized substrates was detected among the various PHB treatments ($P > 0.05$) (n = 3, m = 5). n = number of replicates; m = number of fish sampled per replicate for analysis.

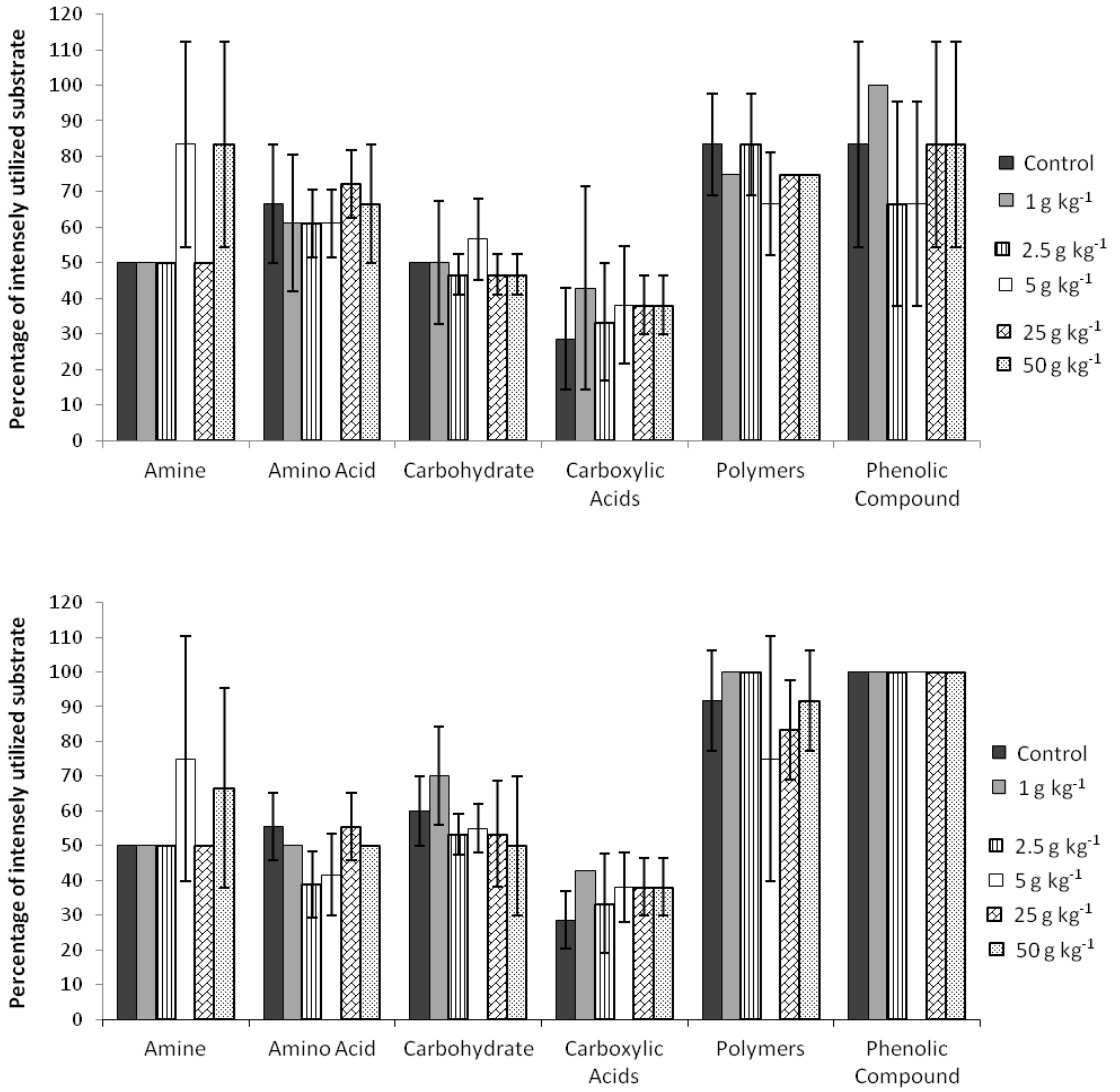


Figure 3.4. Percentage of intensively used substrates (blanked absorbance > AWCD) among biochemically similar substrate classes for intestine sample (upper graph) and faeces sample (lower graph). The bars represent the mean values and error bars represent standard deviation. No significant difference in the substrate utilization was detected among the various PHB treatments ($P > 0.05$) (n = 3, m = 5). n = number of replicates; m = number of fish sampled per replicate for analysis.

Discussion

Fish performance

In general, the PHB-supplemented diets tested in this study improved the average body weight gain of Nile tilapia fingerlings relative to the control group. Several studies have reported a similar growth promoting effect of PHB in aquaculture animals when supplemented at different dietary levels (De Schryver et al., 2010; Defoirdt et al., 2007b; Halet et al., 2007; Nhan et al., 2010). De Schryver et al. (2010) found increases in average body weights of European sea bass juveniles when PHB was dosed in the diet at levels of 20 and 50 g PHB kg^{-1} diet. In this study, the lowest PHB supplementation level of 1 g PHB kg^{-1} diet induced similar results as the higher supplementation levels up to 50 g PHB kg^{-1} diet. This indicates that a low level of PHB supplementation can already provide beneficial growth promoting effects, although this seems to be species specific. This should be highlighted because the use of PHB as a feed supplement does not only depend on the beneficial effects it provides in the animal cultures, but also on its cost effectiveness.

Apart from its beneficial growth promoting effects, PHB had no substantial effect on fish composition as no significant differences were found in the whole body total crude protein and lipid content. Although a trend of increased content in main fatty acid groups was noted, which can be of biological importance (Patterson et al., 2014), also here no significant differences were observed. A previously similar experiment using smaller fish (Nile tilapia juvenile of ~250 mg) showed slightly different results, where dietary PHB increased the whole body total lipid content, but not the crude protein level or fatty acids profile (Situmorang *et al.*, unpublished data). Differences in the influence PHB has on the lipid metabolism at different fish ages was also found by Najdegerami et al. (2013). These authors described that PHB increased the lipid content in sturgeon larvae while a reduction was observed in sturgeon fingerlings. It was suggested that the difference in mode of action of PHB in larvae and fingerlings was different and that this may be related to a

different functionality of the microbiota in the fish gut at different life stages. In this study, it was therefore hypothesized that the growth modulation effect of PHB for the tilapia fingerlings was related to its effect on the functionality of the gut microbiota and a resulting altered feed digestion and nutrients metabolism.

Community-level physiological profile

Distribution of microbial community substrate utilizations

It is widely accepted now that the fish diet modulates the fish intestinal microbiota composition (Austin, 2006; De Schryver et al., 2010; Merrifield et al., 2010; Najdegerami et al., 2012; Neuman et al., 2014; Ringø et al., 1995). Recently, Bolnick et al. (2014) reported that diet-induced changes in intestinal microbiota composition coincided with changes in relative abundance of gene ontology functions involved in digestive system, energy and nucleotide metabolism, and carbohydrate metabolism and transcription. The authors then suggested that dietary changes in intestinal microbiota probably alter metagenomic function and may have consequences for host metabolism and digestion. In this study, the higher Shannon-Wiener index value and lower Gini coefficient for the intestine samples indicate a more diverse metabolic functionality and more equal use of the different substrates by the resident gut MC as compared to the faecal samples that represent the transient gut MC. However, PHB did not seem to have a significant effect on the metabolic properties of neither the resident nor the transient gut MC.

Principal Component Analysis (PCA)

An overlap in coordinate scores was mainly seen for many of the substrates indicating a lesser distinctive pattern between treatments. That is, the more the samples are similar, the more they close to each other in an ordination axes and vice versa (Garland and Mills, 1994). Similar to the previous findings, PCA was not able to reveal distinctive patterns between PHB treatments, as all treatments were found to be highly correlated / similar with each other. Thus, based on the relatively equal levels of utilization of substrates in Biolog EcoPlate, it is suggested

that dietary PHB did not affect fish gut MC, with respect to their functional abilities of utilization of specific substrates.

Substrate richness

CLPP measures functional potential rather than *in situ* activity (Garland et al., 1997). It was, therefore, hazardous to interpret Biolog profiles strictly as a reflection of *in situ* carbon source metabolism. It has nevertheless been reported that a positive response reflects the presence of specific strains capable of using a particular substrate, while a negative response typically indicates the absence of individuals capable of degrading that substrate (Haack et al., 1995). Thus, variations in substrate richness and the number of intensively used substrates per biochemical class are indicative for differences in metabolic diversity of the gut MC between treatments. In this study, as for the other parameters, bacterial populations did not appear to be affected by dietary PHB with respect to their functional abilities to utilized specific biochemical substrate classes. It thus gives the full impression that PHB did not specifically stimulate (some members of) the gut MC to change the digestion pattern of the feed.

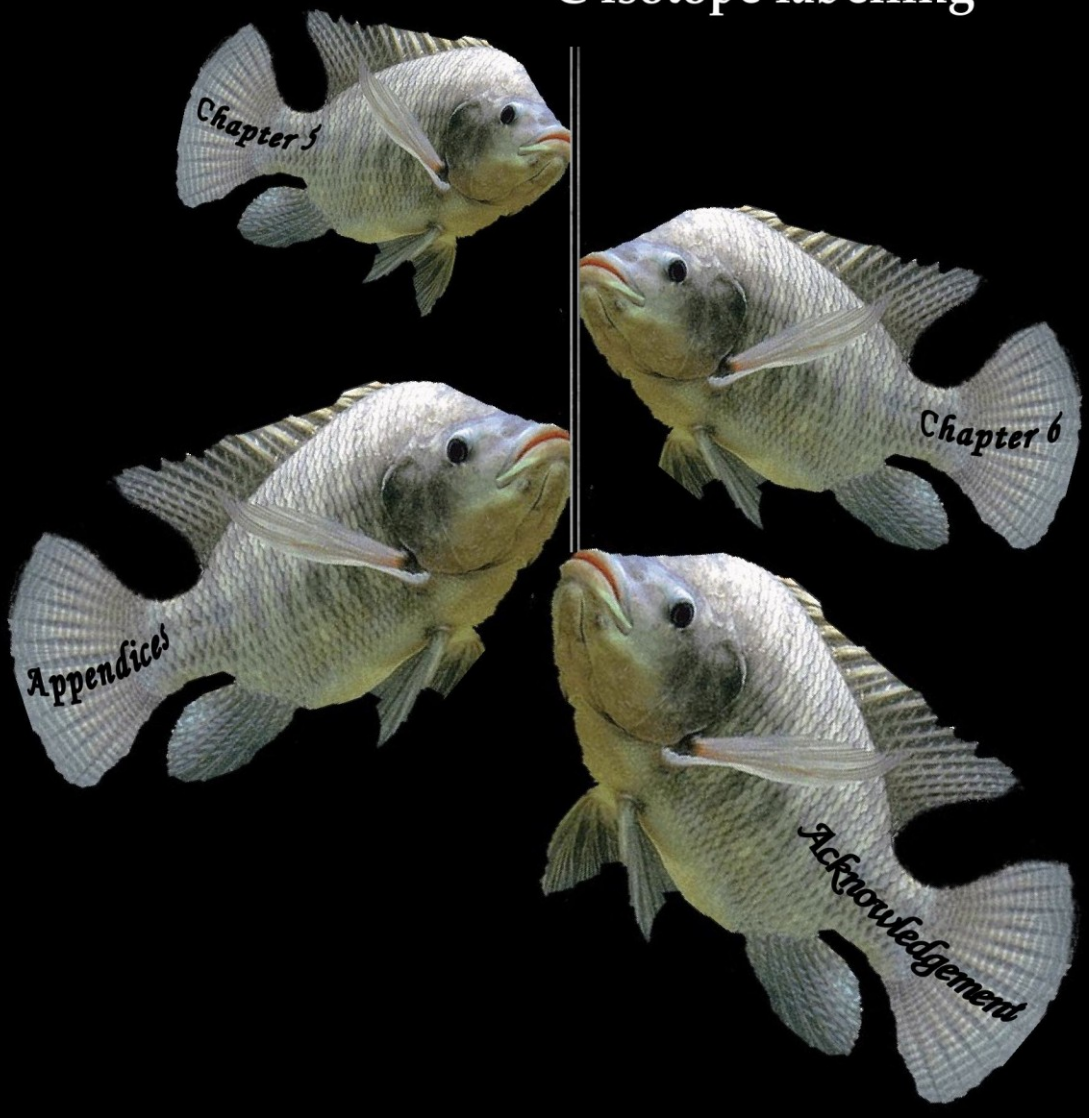
In the Biolog EcoPlate method, the extent of substrate utilization can have a direct correlation to substrate selectivity for inoculated microbial communities (Christian and Lind, 2006). However, it should also be taken into consideration that, as like the other culture-dependent techniques, this method detects only the microorganisms capable of growing on specific, selective media. A proportion of the bacterial population will remain undetected. It might be possible that PHB, irrespective of its doses, changed sub-populations of the microbiota, but that the use of the 31 EcoPlate substrates was not adequate to reveal these changes. In that a functional profiling based on a larger diversity of substrates may be advised. The optimum number and kinds of carbon sources required to profile functional diversities among aquatic microbial communities should further be assessed, e. g., using Biolog GN-/ GP-Microplates or Biolog MT plates.

Conclusions

In general, the current study showed that the addition of PHB in the diet of Nile tilapia fingerlings induced an increased body weight gain of the fish. The CLPP analysis using the Biolog EcoPlate™ method can differentiate the resident (mucosa associated) and the transient (faeces associated) gut MC but it could not detect any differences in either the resident or the transient gut MC functional physiological profile between control and PHB treatment groups. Thus, it can be suggested that the growth promoting effect of PHB was not necessarily related to changes in the functional gut MC profile or its feed digestion pattern. As a consequence, further studies on making the link between PHB supplementation and its growth performing effect remain necessary. Alternatively, CLPP analysis using Biolog Microplate™ containing 95 different substrates might allow better differentiation between control and PHB treatments. Based on this study, it is hypothesized that the growth promoting effect of PHB might be related to the morphological and/or physiological response of the fish, instead of its intestinal microbiota. As for this, advance knowledge on the metabolic fate of PHB in the fish body, e.g. based on isotope tracer study, is critical to elucidate the exact mechanisms of action responsible for the growth promoting effect of PHB.

Chapter 4

The compartmental distribution of poly-β-hydroxybutyrate after ingestion by Nile tilapia fingerling as monitored by stable ^{13}C isotope labelling



Chapter 4

The compartmental distribution of poly-β-hydroxybutyrate after ingestion by Nile tilapia fingerling as monitored by stable ^{13}C isotope labelling

Magdalena Lenny Situmorang[a], Kristof Dierckens[a], Julie Ekasari[b], Linsey Garcia-Gonzales[c], Peter Bossier[a], Peter De Schryver[a]

[a]*Laboratory of Aquaculture & Artemia Reference Center (ARC), Ghent University, Ghent, Belgium*

[b]*Department of Aquaculture, Bogor Agricultural University, Bogor, Indonesia*

[c]*Vlaamse Instelling voor Technologisch Onderzoek (VITO), Mol, Belgium*

Abstract

The biopolymer poly-β-hydroxybutyrate (PHB) is suggested as growth promoter and disease controlling agent for different species of aquaculture organisms. However, the mechanisms of action of PHB and its fate within an animal after ingestion are far from understood. In this study, PHB contained in the bacterium *Ralstonia eutropha* DSM545 was labelled with ^{13}C and dosed in the diet of Nile tilapia (*Oreochromis niloticus*) juveniles during a period of 22 days. The ^{13}C isotopic signature of different tissues (intestine, liver, kidney, heart, spleen, blood, brain, muscle) and the faeces was determined in a time series. Varying $\delta^{13}C$ values among tissues ($P \leq 0.05$) could be detected within 4 h after labelled PHB feeding, reflecting high differences in tissue turnover rates for the compound and/or its degradation product(s). In general, the higher response in isotopic incorporation of PHB in intestine and liver, as well as kidney and spleen, indicate their primary action as metabolically active organs ('source tissues') in PHB uptake and metabolism. The heart rather acts as an 'assimilator tissue', while the blood acts as the 'carrier tissue', and the brain and muscle as the 'sink tissues'. The combination of intestine and liver being important in PHB metabolism and of the fatty acid nature of PHB suggests an association of PHB with the lipid and fatty acid metabolism.

Introduction

Despite all the evidence on the beneficial effects of PHB in growth promotion in aquaculture production (De Schryver et al., 2010; Defoirdt et al., 2007b; Laranja et al., 2014; Najdegerami et al., 2012; Nhan et al., 2010; Sui et al., 2012; Thai et al., 2014), detailed knowledge about the compound's actual mechanism of action remains very limited. In order to understand how PHB stimulates growth and to optimize future PHB application, one should know how PHB distributes in the body once it is ingested. Improved knowledge on the PHB metabolism in the animal body is specifically needed to adequately design future nutritional experiments on PHB. Pure isotope tracers can be applied to evaluate the metabolic fate of specific dietary ingredients in an animal's body under normal feeding and environmental conditions (Viegas et al., 2013). By sampling different tissues in a single individual, stable isotope analysis permits to explore the allocation of the tracer among tissues (Dalerum and Angerbjorn, 2005) while information on the time course of the compound's isotopic signature in the different tissues allows to determine the temporal distribution in the body (Newsome et al., 2007; Phillips and Eldrige, 2006).

The use of stable isotope analysis has been applied in various aquaculture nutrition studies, including the monitoring of the incorporation of live food or compound diet in animal tissue during larviculture production (Schlechtriem et al., 2004; 2005). In this study, PHB contained within the bacterium *Ralstonia eutropha* DSM545 was labelled with the stable isotope ^{13}C and used as a tracer in the diet of Nile tilapia juvenile. The total carbon and $\delta^{13}C$ values of different tissues and the faeces was measured in function of time to trace the carbon originating from the ^{13}C-labelled PHB diet and to determine the compartmental distribution of PHB throughout the fish body after ingestion. The information on the compartmental distribution of PHB is expected to allow to hypothesize about the transfer kinetics of carbon originating from PHB throughout the body as well as about the metabolization of PHB.

Materials and methods

Production of non-labelled and ^{13}C labelled R. eutropha DSM545

R. eutropha DSM 545 (also known as *Cupriavidus necator*) was used as the experimental microorganism. According to the DSMZ website (www.dsmz.de), this strain, a mutant of *C. necator* DSM 529, constitutively expresses glucose-6-phosphate dehydrogenase. A stock culture of *R. eutropha* DSM 545 was stored at -80 °C in 2 mL cryovials containing 0.5 mL of 80 % glycerol (Merck, Germany) and 1 mL of a late exponential-phase liquid culture in Lennox broth (LB) medium (Invitrogen, Life Technologies Europe B.V., Belgium). A 200 µL aliquot of this stock culture was inoculated into 5 mL of LB medium in 15-mL test tubes and cultivated in an orbital shaker (Innova 42, Eppendorf, USA) for 24 h at 30 °C and 200 rpm. Subsequently, 2 mL of this culture was sub-cultured for 24 h at 30 °C and 180 rpm in 100 mL of seeding medium in 500-mL baffled flasks. The seed medium contained 10 g L^{-1} glucose, 3 g L^{-1} $(NH_4)_2SO_4$, 1.5 g L^{-1} KH_2PO_4, 4.47 g L^{-1} $Na_2HPO_4.2H_2O$, 0.2 g L^{-1} $MgSO_4.7H_2O$, and 1 mL L^{-1} trace element solution. The trace element solution had the following composition: 10 g L^{-1} $FeSO_4.7H_2O$, 2.25 g L^{-1} $ZnSO_4.7H_2O$, 1 g L^{-1} $CuSO_4.5H_2O$, 0.5 g L^{-1} $MnSO_4.5H_2O$, 2 g L^{-1} $CaC1_2.2H_2O$, 0.23 g L^{-1} $Na_2B_4O_7.10H_2O$, 0.1 g L^{-1} $(NH_4)_6Mo_7O_{24}$, and 35 % HC1 10 mL L^{-1} and was filter sterilized through a 0.2-µm polyethersulfone filter (Whatman, UK) prior to use. Glucose and $MgSO_4.7H_2O$ were separately autoclaved at 121 °C for 15 minutes. All ingredients were aseptically combined after cooling. Finally, 12.5 % (v/v) of the seed culture was used to inoculate the bioreactor (3 L, Applikon Biotechnology, the Netherlands). The initial working volume of the culture was 700 mL. The pH was maintained at 6.80 by a 2 M H_2SO_4 solution and a 20 % NH_4OH solution, which was replaced by a 5 M NaOH solution during the period of ammonium limitation. The dissolved oxygen (DO) concentration level was regulated at 55 % of air saturation during the biomass growth phase to ensure no PHB accumulation from glucose occurred due to oxygen limitation. For PHB production, DO was kept at 30 % of air saturation during a cascade control strategy consisting of the agitation

speed (850 up to 1000 rpm) and air flow. The cultivation medium initially consisted of 5 g L^{-1} glucose and 3 g L^{-1} $(NH_4)_2SO_4$, 13.3 g L^{-1} KH_2PO_4, 1.2 g L^{-1} $MgSO_4.7H_2O$, 1.87 g L^{-1} citric acid, and 10 mL L^{-1} trace element solution and was prepared similar to the seed medium. Glucose and ammonium (NH_4^+-N) were present according to the mass balance of *R. eutropha* DSM545 (Mozumder et al., 2014) to ensure simultaneous depletion of both substrates. At that time instance, 18 g L^{-1} of ^{13}C labelled glucose (Euroisotop, France) was added aseptically to the cultivation broth. The carbon flux was redirected from biomass to PHB synthesis and the cells started to accumulate PHB from ^{13}C labelled glucose. Samples were taken at regular time intervals and analyzed for the concentration of glucose, ammonium, cell mass (expressed as cell dry mass, CDM) and PHB as previously described (Mozumder et al., 2014; Thai et al., 2014).

Experimental set up

Nile tilapia fingerling with an average body weight of 9.1±0.6 g were produced and reared on commercial diet (500-800 μm; Coppens International, the Netherlands) at the Laboratory of Aquaculture & Artemia Reference Center, Ghent University. During a one week acclimatization period, 70 fingerlings were housed in a 200 L tank supplied with continuous aeration and fed three times a day with a control diet (semi-purified diet without PHB; Table 4.1) at a level of 5 % BW day^{-1} (Ng and Romano, 2013). Daily removal of uneaten feed and faeces, together with 30 % water renewal was done prior to first feeding. A photoperiod of 14L:10D was applied, water temperature was maintained at 26±1 °C and water quality (NO_2^- and NH_4^+) was maintained below 1 mg NO_2^--N L^{-1} and 1 mg NH_4^+-N L^{-1}, respectively.

Following the acclimatization period, fish were transferred to individual 10 L tanks and fed for 3 weeks at 5 % BW day^{-1} with an experimental pre-diet. The pre-diet is the control diet that was supplemented with 5 g kg^{-1} PHB at the expense of cellulose to prepare the fish to the digestion of PHB (referred to as 'preparation period'). As the PHB was present in the lyophilized bacteria at 75 % on cell dry weight the amount of bacteria added in the diet was 6.67 g kg^{-1}. The $\delta^{13}C$ value of

this pre-diet was -19.4 ‰. Fish were fed 3 times daily (8:00, 14:00 and 20:00). On a daily basis, uneaten feed and faeces were removed and water was renewed prior to feeding. The physical environmental conditions during this preparation period were the same as during the acclimatisation period.

Table 4.1. Formulation of basal semi-purified diet (control diet) for Nile tilapia fingerling with initial BW of 9.1±0.6 g

Ingredients	**g kg^{-1}**
Carboxymethyl cellulose (Sigma)	40
Vitamin C (StayC 35% VDS)	0.6
L-methionine (Sigma)	5
Vit + Min mix (VDS)	12.5
L-lysine (Sigma)	15
Choline chloride	1.8
α-cellulose	50
Corn meal (Bio Planet)	180
Fish herring meal (VDS)	200
Corn gluten meal (Sigma)	400
Fish oil (VDS)	20
Soybean oil	75
Vitamin E (95%)	0.1

Uptake and elimination period

In a next phase, the fish were fed with the tracer diet, which was a semi-purified diet containing ^{13}C-labelled PHB for a period of 528 h (22 days). Due to few technical issues (i.e. higher fish growth rate more than expected, and limitation on availability of labelled-PHB), the inclusion level of PHB in the labelled diet had to be reduced from 5 g PHB kg^{-1} diet (as applied in the pre-diet during acclimatization period) to 3 g PHB kg^{-1} diet in order to assure enough food amount needed during the course of the experiment. As the PHB was present in the lyophilized bacteria at 75 % on cell dry weight the amount of bacteria added in the diet was 4 g kg^{-1}. The δ^{13}C value of this labelled diet was 196.8 ‰. The period of 528 h during which fish were fed with the labelled diet is referred to as 'uptake period'.

As a last phase, when the ^{13}C-labelled PHB feeding was stopped after 528 h, fish feeding was continued using the unlabelled pre-diet, for a period of 192 h (8 days). This period when fish were switched back to the unlabelled pre-diet is referred to as 'elimination period'. The daily maintenance and environmental conditions during the uptake and elimination period were identical as during the preparation period.

Sampling and feeding regime

Fish were sampled at specific time points throughout the experiment. The 5 replicate fish to be sampled at each sampling point were randomly assigned using an online random sequence generator (www.random.org). The fish were euthanized using an overdose of MS-222 in ice-cold water and dissected using scissors and forceps to collect the intestine (cleaned from intestinal content), liver, spleen, heart, muscle, kidney, and brain. Between the sampling of each organ, the scissors and forceps were thoroughly cleaned with tap water and dried using a paper cloth. Blood (0.5-1 mL) was collected from the caudal vein using heparin-treated disposable syringe and sterile needle (25-gauge; BD PrecisionGlide®; Becton, Dickinson and Company, USA). Faeces were collected from the tank after dissection of the fish. Each of the samples was dried in a small tin reservoir at 60 °C for 24 h (complete dryness) and cooled in a desiccator to room temperature. After drying, the samples were homogenized with a pestle, transferred into pre-weighed small pressed tin capsules (5 mm x 8 mm; Sercom Ltd, UK) and accurately weighed at 1.0±0.6 mg. The top of the capsules was closed after adding the sample, and the cups were stored at room temperature until ^{13}C isotope analysis.

The different sampling points can be assigned to the different experimental periods:

- *Samples from the preparation period*: 5 fish were sampled at the end of the preparation period, which was just before the start of the uptake period. This sampling point was considered as t = 0 h, and yielded the control samples of the experiment (i.e. natural steady state).

- *Samples from the uptake period*: 5 fish were sampled at 2 h, 4 h, 8 h, 12 h, 24 h, 48 h, 72 h, 96 h, 192 h, 360 h and 528 h relative to the first time being fed with the labelled-diet. The feeding regime during the uptake period depended on the sampling time:
    * Fish sampled at t = 2 h were given a single feeding (at 09:00 of the first sampling day) with the tracer diet at 2 % BW.
    * Fish sampled at t = 4 h were fed two times (at 09:00 and 12:00 of the first sampling day) at a total feeding level of 3 % BW.
    * Fish sampled at t = 8 h were fed three times (at 09:00, 12:00 and 15:00 of the first sampling day) at a total feeding level of 4 % BW.
    * Fish sampled at t = 12 h were fed four times (at 09:00, 12:00, 15:00 and 18:00 of the first sampling day) at a total feeding level of 5 % BW.
    * Fish sampled at 24 h, 48 h, 72 h, 96 h, 192 h, 360 h and 528 h were fed 5 times daily (at 09:00, 12:00, 15:00, 18:00 and 20:00) at a total feeding level of 6 % BW day^{-1}.
- *Samples from the elimination period:* 5 fish were sampled at 24 h and 192 h relative to the restart of being fed again with the unlabelled pre-diet.

The feeding regime during the elimination period was 5 times daily at 09:00, 12:00, 15:00, 18:00 and 20:00, at a total feeding level of 6 % BW day^{-1}.

Isotope Ratio Mass Spectrometry

The samples were analyzed for total carbon and $\delta^{13}C$ at the UC Davis Stable Isotope Facility (Department of Plant Sciences, University of California, USA). Carbon isotope composition analysis was performed by continuous flow isotope ratio mass spectrometry (IRMS; 20-20 mass spectrometer, Sercon, Crewe, UK) after sample combustion to CO_2 at 1000 °C in an elemental analyzer (Sercon GSL). Provisional isotope values obtained from the mass spectrometer were corrected based on working standards distributed at intervals in each analytical run. The included standards were bovine liver ($\delta^{13}C$ = -21.7 ‰, Vienna Pee Dee Belemnite (VPDB)), USGS-41 glutamic acid ($\delta^{13}C$ = 37.6 ‰, VPDB), Nylon 5 ($\delta^{13}C$ = -27.7 ‰,

VPDB) and glutamic acid ($\delta^{13}C$ = -28.8‰, VPDB). Additionally, elemental totals were calculated based on peak area response to calibration standards. The same reference material analyzed over the analysis period was measured with ±0.2 ‰ precision for natural materials and ±0.4 ‰ precision for enriched materials. Stable isotope ratios were presented in delta notation (δ) in parts per million (‰) and calculated as:

$$\delta^{13}C = \left(\left(\frac{R_{sample}}{R_{standard}} \right) - 1 \right) \times 1000$$

, where $\delta^{13}C$ is the stable carbon isotope ratio, while R_{sample} and $R_{standard}$ are the molar ratio of the heavy/light isotope ($^{13}C/^{12}C$) of the sample and the international reference (VPDB); 0.0112372), respectively.

The level of isotopic incorporation into each tissue during the uptake period was calculated as $\Delta\delta^{13}C_{uptake} = \delta^{13}C_{t\text{-}uptake} - \delta^{13}C_{t=0}$, where $\delta^{13}C_{t\text{-}uptake}$ is the $\delta^{13}C$ value of the tissue at time t during the uptake period, and $\delta^{13}C_{t=0}$ is the $\delta^{13}C$ value of the tissue at the beginning of the uptake period (t = 0 h). Similarly, the level of isotopic elimination from each tissue during the elimination period was calculated as $\Delta\delta^{13}C_{elimination} = \delta^{13}C_{t\text{-}elimination} - \delta^{13}C_{t=0'}$, where $\delta^{13}C_{t\text{-}elimination}$ is the $\delta^{13}C$ value of the tissue at time t during the elimination period, and $\delta^{13}C_{t=0'}$ is the $\delta^{13}C$ value of the tissue at the beginning of the elimination period, which was the same as the $\delta^{13}C$ value of the tissue at the end of the uptake period (t = 528 h).

Statistic analyses

Two factors repeated measures ANOVA (RM-ANOVA) was used when data were represented in a cumulative way to evaluate the changes in isotopic ratio of different tissues at multiple time points during the uptake and elimination period. Data sphericity was tested prior the RM-ANOVA by means of the Mauchley's test to check for the assumptions that there is no interaction among times and tissues. One-way analysis of variance (one-way ANOVA) was further used to describe the change-point of each tissue, i.e. the time when the $\delta^{13}C$ value of the tissue was

significantly changed from its initial level. Furthermore, differences in carbon isotope incorporation ($\Delta\delta^{13}C_{uptake}$) and elimination ($\Delta\delta^{13}C_{elimination}$) among tissues at multiple time points during the uptake and elimination period, respectively, were tested by means of one-way ANOVA. A posteriori comparisons were carried out with the Duncan's multiple range test using 95 % confidence limits. All statistical analyses were performed using the STATISTICA statistical software (Version 7.0).

Results

Isotope ratio of tissues during uptake period

The mean $\delta^{13}C$ values of the different tissues at the start of the experiment reflected the $\delta^{13}C$ value of the unlabelled pre-diet (-19.4 ‰). Following the start of the uptake period, the faeces showed a very high increase in the $\delta^{13}C$ values already from 2 h onwards. The $\delta^{13}C$ value of the faeces was levelling up to the $\delta^{13}C$ value of the labelled diet (196.8 ‰) at 2 h (Table 4.2). The $\delta^{13}C$ values of all tissues also significantly increased during the uptake period. It can be observed that the rate at which carbon was derived from the PHB diet and was incorporated during the uptake period was highly variable among tissues (Fig. 4.1). The overall trend showed higher and faster isotope shifting in the intestine, liver, kidney and spleen compared to the heart, brain, blood and muscle. The intestine and liver showed a fast increase in $\delta^{13}C$ value, resulting in an asymptotic like curve which seems to be levelling off towards the end of the uptake period (Fig. 4.1.a-b). Similarly, the kidney and spleen also showed a fast increase in $\delta^{13}C$ content following a curved trend but which did not seem to be levelling off yet at the end of the uptake period (Fig. 4.1.c-d). A more linearly increase in $\delta^{13}C$ values with a rather high slope was shown by the heart (Fig. 4.1.e), while a linear response with a lower slope was shown by the blood, brain and muscle (Fig. 4.1.f-h).

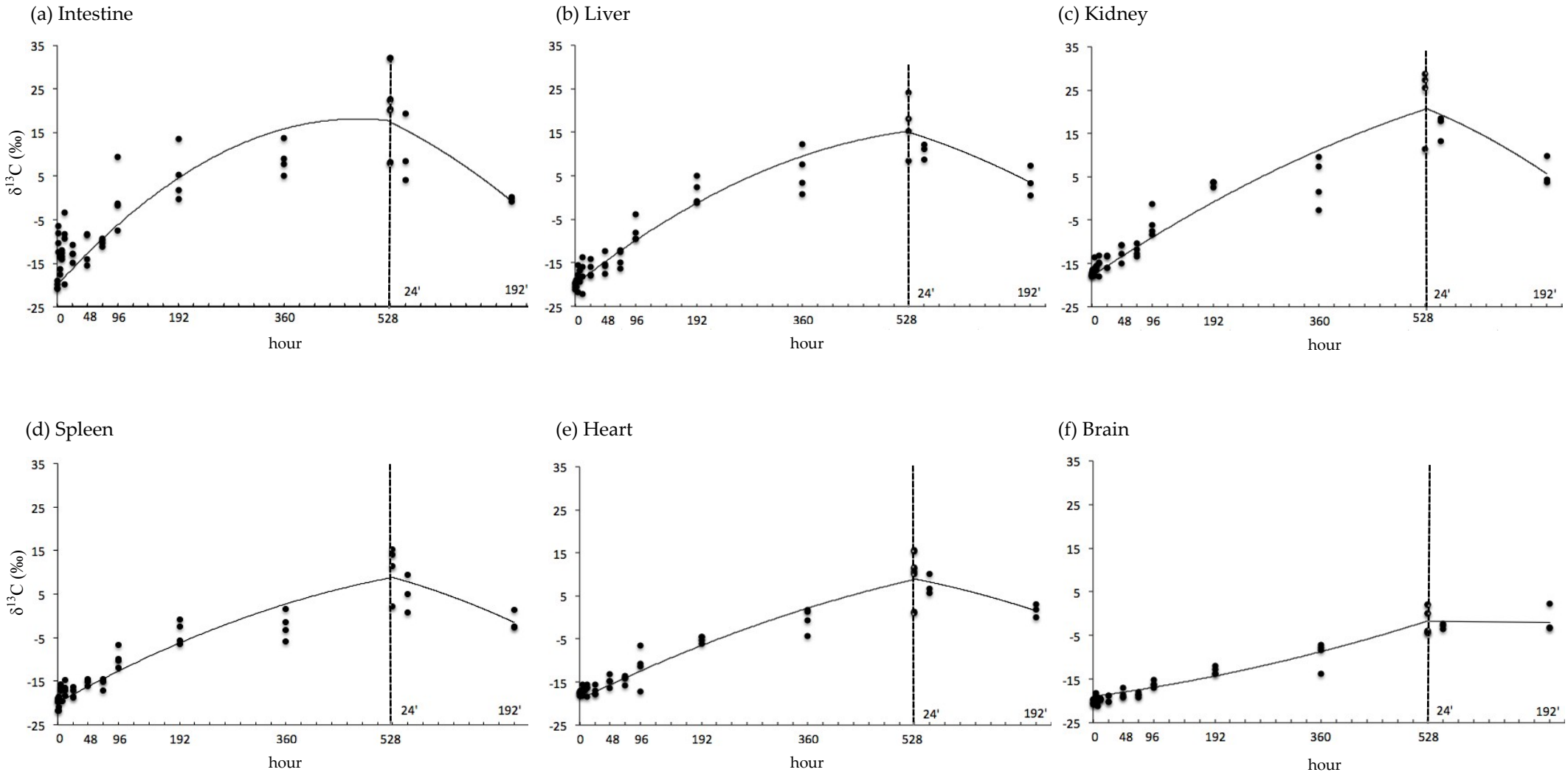


Figure 4.1. $\delta^{13}C$ values (‰) of different fish tissues (a – i) and faeces during the uptake period (up to 528 h), followed with the elimination period (up to 192 h) – *part 1*. The vertical dashed line separates the two experimental periods. Time is in hours after the first labelled feeding in the uptake period or after the first unlabelled pre-diet feeding in the elimination period. Filled circles are data observations; solid line / curve is polynomial trend line of data observations.

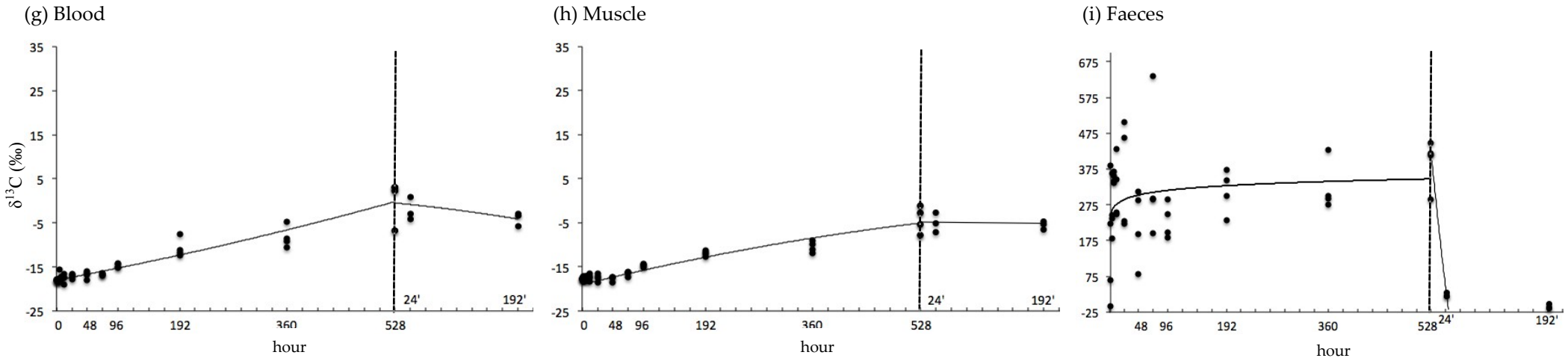


Figure 4.1. $\delta^{13}C$ values (‰) of different fish tissues (a – i) and faeces during the uptake period (up to 528 h), followed with the elimination period (up to 192 h) – *part 2*. The vertical dashed line separates the two experimental periods. Time is in hours after the first labelled feeding in the uptake period or after the first unlabelled pre-diet feeding in the elimination period. Filled circles are data observations; solid line / curve is polynomial trend line of data observations.

Tissue change-points and isotope incorporation levels during uptake period

The results of one-way ANOVA on the differences in $\delta^{13}C$ values of each tissue during the uptake period showed that there are various change-points between tissues (Table 4.2). Based on the change-points, different tissues can be categorized into four groups: (1) intestine; 12 h, (2) liver, kidney, and spleen; 48 h, (3) heart, brain, muscle; 96 h, and (4) blood; 192 h. This suggests that there are differences in the isotopic incorporation rate among tissues during the uptake period. Furthermore, the results of one-way ANOVA on the temporal differences in the isotopic incorporation levels (i.e. $\Delta\delta^{13}C$ values) among tissues during this uptake period, showed that the significant differences in isotopic incorporation levels among tissues were already detected from 2 h onwards (Table 4.3). Overall, in addition to the earliest change-point (at 12 h), the intestine showed the highest $\Delta\delta^{13}C_{uptake}$ values among all tissues, with significant differences when compared to the heart, blood, brain, and muscle ($P \leq 0.05$) at all times during the uptake period; while the differences with the liver, kidney and spleen were not always significant (Table 4.3). In addition to the similar change-point (at 48 h), the liver, kidney and spleen also showed similar $\Delta\delta^{13}C_{uptake}$ values during most of the uptake period (the $\Delta\delta^{13}C_{uptake}$ value of the spleen were significantly lower than of the liver and kidney at time 192 h and 360 h; no significant differences in $\Delta\delta^{13}C_{uptake}$ value were found between the liver, kidney and spleen at all other time points). A different pattern was observed for the heart, brain, and muscle; even though they showed similar $\Delta\delta^{13}C_{uptake}$ values at their change-point (at 96 h) but the $\Delta\delta^{13}C_{uptake}$ values of the heart were found to be significantly higher than the brain and muscle from 192 h onwards. As for the blood, even though it showed the slowest change-point (at 192 h), its $\Delta\delta^{13}C_{uptake}$ values were similar with the brain and muscle during most of the uptake period (the $\Delta\delta^{13}C_{uptake}$ value of the blood was significantly higher than of the muscle at time 48 h; no other significant differences in $\Delta\delta^{13}C_{uptake}$ value were found between the blood, brain and muscle at all other time points). At the end of the uptake period, the highest $\Delta\delta^{13}C_{uptake}$ values were obtained by the intestine and

kidney, which were higher than the liver and spleen ($P > 0.05$), and the heart ($P \leq 0.05$). Lastly, the lowest $\Delta\delta^{13}C_{uptake}$ values among all tissues were observed in the blood, brain, and muscle ($P \leq 0.05$) (Table 4.3).

Tissue isotopic elimination levels during elimination period

For the elimination period, differences in the isotopic elimination levels ($\Delta\delta^{13}C_{elimination}$) between different tissues were already observed within the first 24 h of elimination period. At the end of the elimination period, the highest $\Delta\delta^{13}C_{elimination}$ value was obtained by the intestine. A lower $\Delta\delta^{13}C_{elimination}$ value was observed in the liver and kidney, which then followed by $\Delta\delta^{13}C_{elimination}$ value of the spleen and heart. The lowest $\Delta\delta^{13}C_{elimination}$ values were observed in the brain and muscle (Table 4.3).

Table 4.2. $\delta^{13}C$ values of different tissues, as well as faeces, during trace experiment (Mean±SD). Five fish were analyzed at each sampling time). Different letters in columns indicate significant difference in $\delta^{13}C$ values between sampling times for each tissue during the uptake period (one-way ANOVA; $P \leq 0.05$)

Time (h) during uptake period	**$\delta^{13}C$ (‰)**								
	Intestine	Kidney	Liver	Spleen	Heart	Blood	Brain	Muscle	Faeces
0	-20.0±0.8^{a}	-17.6±0.4^{a}	-20.3±0.7^{a}	-20.0±1.2^{a}	-17.7±0.4^{a}	-18.0±0.2^{a}	-20.1±0.5^{a}	-17.7±0.2^{a}	-19.5±0.9^{a}
2	*-9.0±2.6**	-16.8±0.4^{ab}	-19.9±0.8^{a}	-20.1±1.0^{a}	-17.1±0.2^{a}	-18.2±0.1^{a}	-20.1±0.6^{a}	-17.7±0.4^{a}	166.6±174.9^{b}
4	-15.2±2.1^{ab}	-16.1±1.8^{ab}	-18.5±2.6^{ab}	-17.2±1.6^{ab}	-17.0±1.1^{a}	-17.4±1.2^{a}	-19.1±0.6^{a}	-17.5±0.3^{a}	256.5±75.9^{bc}
8	-13.0±0.9^{ab}	-15.9±0.3^{ab}	-17.8±1.3^{ab}	-17.5±1.4^{ab}	-16.8±0.3^{a}	-17.6±0.2^{a}	-20.0±0.8^{a}	-17.5±0.5^{a}	350.1±14.4^{c}
12	-10.2±6.9^{b}	-15.3±2.0^{ab}	-17.4±3.6^{ab}	-16.7±1.5^{ab}	-16.6±1.2^{a}	-17.6±1.0^{a}	-19.6±0.2^{a}	-17.4±0.7^{a}	319.1±86.5^{bc}
24	-12.8±1.7^{b}	-14.6±1.6^{ab}	-16.4±1.7^{ab}	-17.7±1.2^{ab}	-17.0±1.1^{a}	-17.1±0.5^{a}	-19.5±0.8^{a}	-17.5±0.8^{a}	354.5±150.9^{c}
48	-11.5±3.7^{b}	-12.3±2.1^{b}	-15.2±2.2^{b}	-15.1±0.7^{b}	-14.8±1.3^{ab}	-16.8±0.8^{a}	-18.4±1.1^{a}	-17.7±0.6^{a}	218.2±104.9^{bc}
72	-10.1±0.8^{b}	-12.1±1.3^{b}	-13.9±2.0^{b}	-15.4±1.2^{b}	-14.4±1.0^{ab}	-16.7±0.3^{a}	-18.6±0.5^{a}	-16.5±0.5^{a}	353.4±192.7^{c}
96	0.2±7.0^{c}	-5.9±3.1^{c}	-7.7±2.6^{c}	-9.7±2.2^{c}	-11.4±4.4^{b}	-14.7±0.5^{ab}	-16.2±0.8^{b}	-14.6±0.3^{b}	230.0±94.4^{bc}
192	5.1±6.0^{cd}	3.4±0.6^{d}	1.3±2.9^{d}	-3.8±2.6^{d}	-5.2±0.8^{c}	-10.7±2.2^{bc}	-13.1±0.9^{c}	-11.9±0.6^{c}	311.7±61.4^{bc}
360	8.9±3.6^{d}	4.0±5.6^{d}	6.0±5.1^{e}	-2.2±3.2^{d}	-0.5±2.7^{d}	-8.3±2.4^{c}	-9.3±3.0^{d}	-10.4±1.3^{d}	324.1±69.6^{bc}
528	20.6±9.8^{e}	23.3±8.0^{e}	16.5±6.5^{f}	10.7±5.9^{e}	9.8±6.1^{e}	0.4±4.8^{d}	-1.5±3.1^{e}	-4.3±2.9^{e}	392.4±70.4^{c}

*This data point is considered as outlier as it does not well correspond with the next sampling point. Sample might be contaminated with ^{13}C-diet particle that were not rinsed completely from the intestine tissue during sampling.

Table 4.3. $\Delta\delta^{13}C$ values of different tissues, as well as faeces, during the uptake and elimination period (Mean±SD). Five fish were analyzed at each sampling time. Different lower case letters in rows indicate significant difference in $\Delta\delta^{13}C_{uptake}$ values among tissues at a specific time during the uptake period (one-way ANOVA; $P \leq 0.05$). Different upper case letters in rows indicate significant difference in $\Delta\delta^{13}C_{elimination}$ values among tissues (excluding faeces) at a specific time during the elimination period (one-way ANOVA; $P \leq 0.05$).

Time (h) during uptake period	$\Delta\delta^{13}C_{uptake}$ (‰)								
	Intestine	Kidney	Liver	Spleen	Heart	Blood	Brain	Muscle	Faeces
2	*10.74±2.58*[*]	0.83±0.44^{a}	0.58±0.70^{ab}	0.63±0.75^{ab}	0.61±0.16^{a}	0.00±0.00^{b}	0.23±0.30^{ab}	0.17±0.24^{ab}	186.09±174.92
4	4.81±2.08^{a}	1.65±1.70^{bc}	2.18±2.03^{abc}	2.79±1.63^{ab}	0.81±0.92^{bc}	0.69±1.12^{bc}	1.09±0.63^{bc}	0.24±0.24^{c}	276.00±75.92
8	7.00±0.99^{a}	1.79±0.33^{bc}	2.45±1.34^{b}	2.49±1.36^{b}	0.90±0.34^{cd}	0.40±0.24^{d}	0.37±0.46^{d}	0.29±0.34^{d}	369.61±14.37
12	9.86±6.90^{a}	2.48±1.82^{b}	3.31±2.84^{b}	3.31±1.49^{ab}	1.21±0.87^{b}	0.67±0.55^{b}	0.53±0.21^{b}	0.48±0.49^{b}	338.52±86.51
24	7.19±1.66^{a}	3.05±1.62^{b}	3.87±1.75^{b}	2.29±1.19^{bc}	0.71±1.03^{d}	0.92±0.53^{cd}	0.63±0.71^{d}	0.46±0.48^{d}	374.00±150.9
48	8.50±3.73^{a}	5.30±2.05^{ab}	5.06±2.18^{ab}	4.87±0.75^{ab}	2.87±1.31^{bc}	1.21±0.84^{c}	1.69±1.05^{c}	0.26±0.14^{d}	237.67±104.9
72	9.92±0.75^{a}	5.59±1.35^{b}	6.36±2.00^{b}	4.59±1.15^{bc}	3.27±1.00^{c}	1.32±0.25^{d}	1.54±0.48^{d}	1.20±0.52^{d}	372.89±192.7
96	19.81±7.03^{a}	11.80±3.12^{b}	12.59±2.65^{b}	10.30±2.17^{b}	6.25±4.40^{c}	3.31±0.50^{c}	3.97±0.79^{c}	3.09±0.29^{c}	249.48±49.36
192	25.09±6.02^{a}	21.10±0.58^{a}	21.61±2.95^{a}	16.14±2.58^{b}	12.52±0.77^{c}	7.29±2.22^{d}	7.05±0.91^{d}	5.79±0.62^{d}	331.15±61.39
360	28.92±3.59^{a}	21.64±5.58^{b}	26.29±5.08^{ab}	17.77±3.17^{c}	17.21±2.73^{c}	9.76±2.42^{d}	10.87±3.03^{d}	7.30±1.29^{d}	343.53±69.58
528	40.63±9.85^{a}	40.93±8.01^{a}	36.80±6.51^{ab}	30.73±5.90^{ab}	27.51±6.08^{b}	18.40±4.81^{c}	18.58±3.07^{c}	13.45±2.93^{c}	411.84±70.42
Time (h) during elimination period	$\Delta\delta^{13}C_{elimination}$ (‰)								
	Intestine	Kidney	Liver	Spleen	Heart	Blood	Brain	Muscle	Faeces
24	10.2±7.9^{A}	6.3±2.9^{AB}	6.7±1.8^{AB}	5.7±4.4^{AB}	2.2±2.0^{B}	2.7±2.4^{B}	1.3±0.6^{B}	1.3±1.5^{B}	-394.55±6.04
196	21.0±0.6^{A}	17.1±3.3^{B}	13.7±3.5^{BC}	12.0±2.3^{C}	7.9±1.5^{CD}	4.4±1.5^{DE}	1.1±1.0^{E}	1.3±0.9^{E}	-427.04±5.19

[*]This data point is considered as outlier as it does not well correspond with the next sampling points. Sample might be contaminated with ^{13}C-diet particle that were not rinsed completely from the intestine tissue during sampling

Discussion

A tracer experiment was used to measure differences among tissues in ^{13}C incorporation in Nile tilapia juveniles with the aim of investigating the compartmental distribution of PHB after ingestion. In this study, the labelled PHB might have been relatively low digestible, as indicated by the high $\Delta\delta^{13}C_{uptake}$ values of the faeces at 2 h until the end of the uptake period where the $\Delta\delta^{13}C_{uptake}$ values reached up to 2.5 times the $\delta^{13}C$ value of the labelled diet, which showed that high amounts of ingested PHB-C ended up in the faeces and made the faeces very highly enriched. Nevertheless, this study succeeded to be the first study that shows PHB is actually metabolized as a dietary compound and contributes to tissue formation. The overall results showed a faster and higher incorporation of PHB-C in the intestine, liver, spleen and kidney as compared to the heart, blood, brain and muscle. The turnover rate of a tissue for a specific element (such as carbon) gives an indication of the metabolic activity of this organ for that compound (Cerling et al., 2007). Highly metabolically active tissues (i.e. splanchnic organs including intestine and liver) have high turnover rates reflecting the balance between uptake of recent dietary inputs for metabolism and growth and output of metabolic products. Other tissues exhibit a much lower turnover rate (i.e. structural elements including muscle and red blood cells) reflecting mainly the incorportation of dietary inputs in the tissue over longer time periods (Ambrose, 2000; Carleton et al., 2008; Dalerum and Angerbjorn, 2005; Gannes et al., 1998; Martinez Del Rio et al., 2009; Pearson et al., 2003; Podlesak et al., 2005; Voigt et al., 2003; Waterlow, 2006). In this study, the exact turnover rate of PHB-C in each tissue could not be determined because its incorporation in all tissues was still progressing at the end of the trial while an equilibrium level is needed for the calculation of the tissue turnover rates (Cerling et al., 2007). However, based on varying change-points and isotopic incorporation levels among tissues observed in this study, a simulation can be made of the circulation of PHB-C in the body.

The observed compartmental distribution of PHB-C is related to the nutrient flow in the animal body (Brindley, 1984; Halver and Hardy, 2002) After ingestion of the PHB diet, dietary ingredients are digested and absorbed by the intestine (Halver and Hardy, 2002). The processes of digestion occur in the intestinal lumen, where complex compounds in the diet (lipids, proteins, carbohydrates) are reduced into smaller 'building blocks' of these compounds (fatty acids, amino acids, monosaccharides) (Buddington and Kuz'mina, 2000). The earliest change-point (at 12 h) and high $\Delta\delta^{13}C_{uptake}$ values showed by the intestine indicate a high level of intestinal activity in the absorption and digestion of PHB-C during the uptake period.

Digested nutrients absorbed by the intestine are then transported to the blood for which there are two routes; the lymphatic vessels of the intestine and the veins of the portal system (Brindley, 1984). Substances such as long-chain fatty acids, phospholipids and cholesterols are mainly transported by the lymphatic system and enter the systemic circulation directly (Sheridan and Friedlander, 1985; Zapata et al., 2006). Other substances, including the short-chain fatty acids, monosaccharides, and amino acids, are transported via the hepatic portal vein, where the spleen is also attached (Buddington and Kuz'mina, 2000), to the liver where they undergo metabolic transformation before entering the systemic circulation (Brindley, 1984; Halver and Hardy, 2002). The liver plays an important role in the lipogenesis and is responsible for the production of bile that emulsifies lipid to aid its digestion, as well as storage site of lipid (Ballantyne, 2014; Buddington and Kuz'mina, 2000). In this study, the high isotopic incorporation in the liver indicates that PHB metabolites are not only metabolized but also taken up by the liver. Furthermore, the high metabolic activity of the liver suggests that the metabolism of PHB is associated with the fatty acid metabolism. Based on this observation, two hypotheses are formulated. Firstly, we hypothesize that PHB is metabolized into the short-chain fatty acid, i.e. butyrate that is one of the common FAs used as carbon source in PHB synthesis by PHB-producing bacteria (Hu et al., 1997; Shimizu et al., 1993). It is reported that although butyrate is greatly

metabolized by the intestinal epithelium (Ballantyne, 2014; Bauermeister and Sargent, 1979; Buddington and Kuz'mina, 2000; Titus and Ahearn, 1988), a certain amount is also absorbed into the portal blood (Velázquez et al., 1997) and taken up by the liver in rat *in vivo* (Bloemen et al., 2009; Demigné et al., 1986). Secondly, we hypothesize that PHB metabolism results in the release of ketone body β-hydroxybutyrate (β-HB). In the liver, fatty acids can also be oxidized into ketone bodies (Halver and Hardy, 2002). Ketone bodies formation from butyrate has been demonstrated in liver slices of rainbow trout and eel (Phillips and Hird, 1977).

From the liver, PHB metabolites (i.e. ketone body β-HB) can be diverted to the gall bladder followed by secretion into the intestine or alternatively returned back in the blood and eliminated by the kidney (Buddington and Kuz'mina, 2000). The kidney filters the blood and also acts as the primary excretory organ (Buddington and Kuz'mina, 2000). In this study, the high $\Delta\delta^{13}C_{uptake}$ values of the kidney indicate its high activity in filtering the blood containing PHB-C. The slow change-point (only at 192 h) and low isotopic incorporation level of the blood is likely related to its function as transport medium of PHB-C, which characterized by a constant uptake and release of PHB-C. This suggests that the blood acts as a 'carrier tissue' for PHB metabolites (i.e. β-HB). Several studies reported that trace amounts of β-HB were found in blood samples, showing the function of blood in the circulation of the ketone body (Hemre et al., 1991; Zammit and Newsholme, 1979). The released ketone bodies are then utilized mostly by the heart (Hemre et al., 1991), but the brain and muscle are also capable to use ketone bodies as their fuels (Beis et al., 1980; Magnoni et al., 2001; Soengas and Aldegunde, 2002). The intermediate level of isotopic incorporation in the heart suggests that the heart acts as 'assimilator tissue' that receives and utilizes the PHB metabolites. On the other hand, the slow and low isotopic incorporation in the brain and muscle indicate their minor response in the uptake of PHB metabolites, suggesting that they rather act as 'sink tissues', which also receive and utilize the PHB metabolites but at lower level.

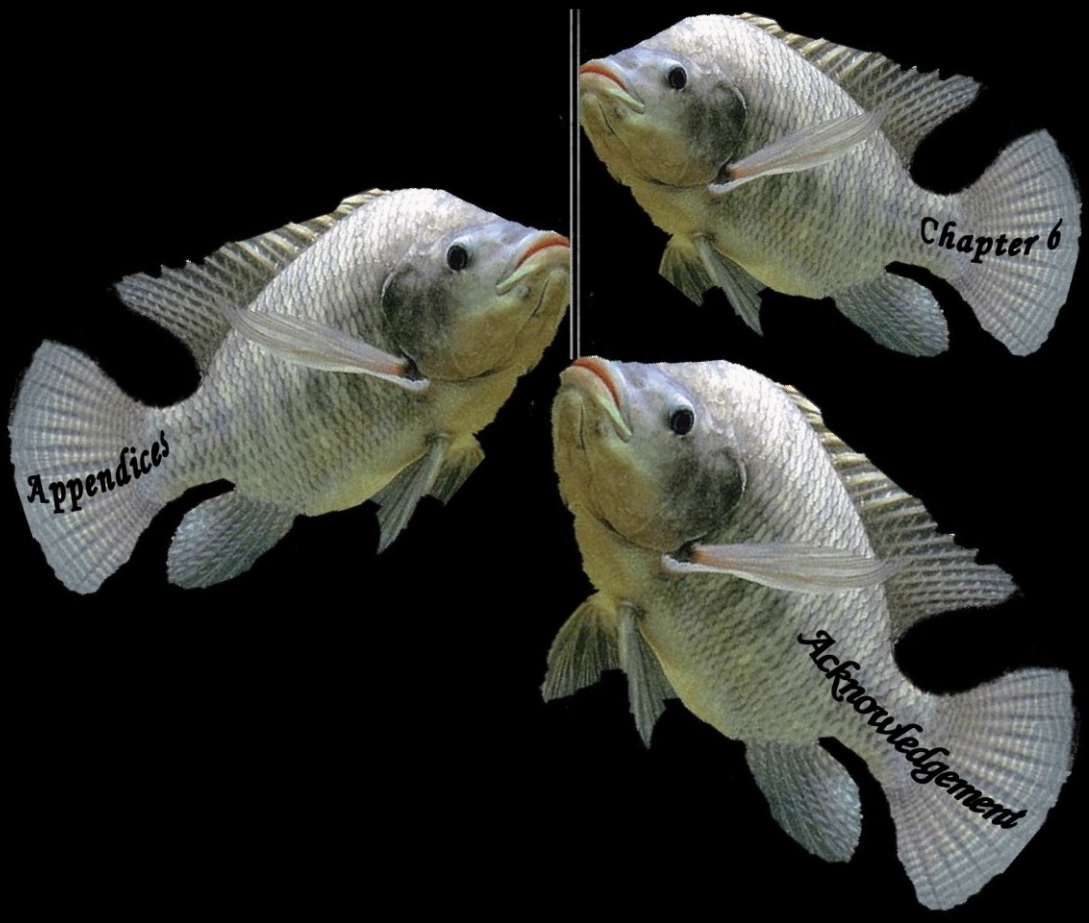
At the end of the elimination period, the intestine, kidney, liver, and spleen showed higher isotopic elimination level (higher $\Delta\delta^{13}C_{elimination}$ values) when compared to the heart, blood, brain and muscle. This further emphasizes the primary response of these major metabolically active organs for the PHB metabolism. The lower isotopic elimination level observed in the heart again indicates its function as an 'assimilator tissue'. Furthermore, the low isotopic elimination levels observed in the blood, brain and muscle again indicate their low response in PHB metabolism due to their suggested function as a 'carrier tissue' (i.e. blood) or 'sink tissues' (i.e. brain and muscle).

Conclusions

In conclusion, the compartmental distribution of PHB in the fish body can be determined through the carbon stable isotope incorporation. The overall results suggest that the intestine and liver, as well as kidney and spleen, are the major metabolically active organs ('source tissues') in PHB uptake and metabolism. It is also suggested that the heart rather acts as an 'assimilator tissue', while the blood acts as the 'carrier tissue', with the brain and muscle as the 'sink tissues'. The combination of intestine and liver being important in PHB metabolism and of the fatty acid nature of PHB suggests an association of PHB with the lipid and fatty acid metabolism. It is hypothesized that once it is ingested, the PHB is digested, absorbed, and metabolized, resulting in the release of SCFA and ketone body β-HB production. These results are especially of interest for a complete elucidation of the PHB metabolic pathway in order to explore the full potential of PHB as an ecologically and economically sustainable alternative as growth modulator and anti-infective strategy for aquaculture.

Chapter 5

Bacterial challenge test for gnotobiotic Nile tilapia larvae: Development and use in poly-β-hydroxybutyrate study



Chapter 5

Bacterial challenge test for gnotobiotic Nile tilapia larvae: Development and use in poly-β-hydroxybutyrate study

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[a] *Laboratory of Aquaculture & Artemia Reference Center (ARC), Ghent University, Belgium*

[b] *School of Life Sciences and Technology, Bandung Institute of Technology, Indonesia*

[c] *Department of Animal Science and Production, Sokoine University of Agriculture, Tanzania*

[d] *Department of Industrial Sciences, Technologies and Biotechnology, Odisee University College Association KU Leuven, Belgium*

Abstract

The use of PHB in the protection of the host against bacterial infections and in increasing the survival and growth performance has been studied in different aquatic animals and diet formulations. To study the treatment of infectious diseases in Nile tilapia, the availability of a method of experimental infection leading to reproducible results in survival is important. In pathogen-screening tests, four candidate pathogenic bacterial strains, *Edwardsiella ictaluri* gly09, *E. ictaluri* gly10, *E. tarda* LMG2793 and *Streptococcus agalactiae* LMG15977 were individually tested on xenic Nile tilapia larvae. Only *Edwardsiella* strains delivered via *Artemia* nauplii, with or without additional pathogen delivery via the culture water, led to a significant increase in mortality of the fish larvae. Based on an antibiotics resistant *E. ictaluri* gly09R strain, a gnotobiotic Nile tilapia larval model system was developed, providing a research tool to investigate the protective effect of poly-β-hydroxybutyrate (PHB) under controlled conditions. A double disinfection procedure using hydrogen peroxide (2000 mg L^{-1} for 10 min) and sodium hypochlorite solution (75 mg L^{-1} active chlorine for 2 min) was applied on the fish eggs. A cocktail of antibiotics (ampicillin, rifampicin, kanamycin, trimethoprim and gentamycin each at 10 mg L^{-1}) and antifungal agents (Amphotericin-B and Fluorescent Brightener-28 at 0.5 mg L^{-1} and 25 mg L^{-1}, respectively) were used to maintain gnotobiotic conditions. The feeding of the challenged gnotobiotic larvae with PHB-enriched *Artemia* nauplii resulted in a 20 % higher mean survival when compared to the challenged control treatment. This indicated that PHB is also effective as an antimicrobial agent for application in Nile tilapia larviculture.

Introduction

Tilapia (*Oreochromis* spp.) are an important group of freshwater aquaculture animals due to their ease of culture under a wide range of environmental conditions and their relatively high resistance to environmental stressors when compared to other cultured finfish species (Welker and Lim, 2011). Under intensive culture conditions, however, significant losses can still occur as tilapia can become infected with a wide range of viral, parasitic, fungal and especially bacterial pathogens. Bacteria of the genus *Edwardsiella,* including *E. ictaluri* and *E. tarda*, have been described as strong pathogens that cause severe economic losses in both freshwater and marine aquaculture in many countries (Evans et al., 2011; Ewing et al., 1965; Hawke et al., 1981). *E. ictaluri,* the causative agent of enteric septicemia of catfish (ESC), is also known to infect and cause mortality in Nile tilapia fingerlings (Plumb and Sanchez, 1983; Soto et al., 2012). *E. tarda,* which is a common bacterium in freshwater environments, has been reported to infect and cause disease in red tilapia (Iregui et al., 2012).

The use of antibiotics for the control of bacterial diseases in aquaculture has resulted in the development and spread of antibiotic resistance, leading to ineffective treatment for some diseases (Defoirdt et al., 2011). This has made researchers examine other mechanisms for the control of bacterial diseases in fish. Several studies have confirmed the role of PHB in the protection of the host against bacterial infections and in increasing the survival and growth performance in different aquatic animals and formulations (Defoirdt et al., 2007a; Defoirdt et al., 2011; Nhan et al., 2010; Sui et al., 2012; Thai et al., 2014).

Overall, the previous studies of PHB in fish and shrimp culture were done in open (or conventional) rearing systems, implying that the composition of the gastrointestinal (GI) MC in the animals may be very dynamic. The composition of the GI microbiota can be influenced by host genotype (Kostic et al., 2013; Spor et al., 2011), environmental conditions and stochastic factors (Fjellheim et al., 2012; Verschuere et al., 1999). This often complicates the study of host-microbial

interactions, which can be important when the unbiased action of a disease treatment is being investigated. Thus, a key strategy in studying host-microbial interactions is to first determine interactions under axenic conditions, and then further evaluate the effects of a single population or defined populations of microbes or specified compounds added under gnotobiotic conditions (Gordon and Pesti, 1971). The use of gnotobiotic organisms leads to an increased control of variables, enhanced reproducibility of results and more accurate experimental designs (Coates, 1975; Marques et al., 2005) and thus is an excellent tool to extend the understanding of the mechanisms involved in host-microbe interactions (Marques et al., 2006).

The aims of the present work were to (i) screen candidate pathogenic bacteria strains for xenic / conventional Nile tilapia *O. niloticus* larvae, (ii) develop a standardized gnotobiotic Nile tilapia larvae culture system to facilitate the studies of host–microbe interactions, (iii) develop a standardized bacterial challenge test for gnotobiotic Nile tilapia larvae, and (iv) employ the developed bacterial challenge test for gnotobiotic Nile tilapia larvae to evaluate the use of PHB and its effects on fish disease resistance. To our knowledge, this is the first study on a gnotobiotic food chain consisting of Nile tilapia larvae and *Artemia* nauplii.

Materials and Methods

Pathogen screening test on xenic Nile tilapia larvae

Larvae production

Nile tilapia broodstock that ranged from 120 to 150 g wet weight and 21 to 23 cm total length were naturally bred in our laboratory to provide tilapia larvae. Three days after hatching (DAH) larvae were collected from the mouth of brooding females and pooled. Upon collection, the pooled larvae were acclimatized in a 30 L aquarium for 6 d at a temperature of 27±1 °C until the start of xenic bacterial challenge. Filtered synthetic freshwater containing 96 mg L^{-1} $NaHCO_3$, 60 mg L^{-1}

$CaSO_4.2H_2O$, 60 mg L^{-1} $MgSO_4$ and 4 mg L^{-1} KCl (EPA, 2002) was used as fish culture water.

Bacterial strains and culture conditions

Streptococcus agalactiae LMG15977 and two strains of *E. ictaluri*, referred to as gly09 and gly10, were tested in the first bacterial pathogen screening test (experiment 1a). Another *Edwardsiella* species, *E. tarda* LMG2793, was tested along with the 2 *E. ictaluri* strains in a second pathogen screening test (experiment 1b). Both *E. ictaluri* strains were obtained from the Laboratory of Aquaculture & Artemia Reference Center, Ghent University, Belgium. These strains were isolated from ichthyophthriasis (white spot disease) infected striped catfish (*Pangasius hypophthalmus*). *S. agalactiae* LMG15977, which was previously isolated from Nile tilapia brain, was obtained from the Belgian Coordinated Collections of Microorganisms (BCCM/LMG), Laboratory of Microbiology, Ghent University, Belgium. Isolate *E. tarda* LMG2793 was provided by the Laboratory of Veterinary Bacteriology and Mycology, Ghent University, Belgium. This isolate was obtained from human faeces.

The strains were stored in Brain Heart Infusion (BHI) broth (FLUKA, Sigma-Aldrich, USA) supplemented with 20 % (*v*/*v*) glycerol at -80 °C. *Edwardsiella* strains were grown in BHI broth and incubated overnight on a horizontal shaker at 160 rpm at 27 °C. *S. agalactiae* LMG15977 was grown in BHI broth and incubated overnight on a horizontal shaker at 180 rpm at 37 °C.

Bacterial challenge procedure for xenic Nile tilapia larvae

Candidate pathogenic bacteria strains were tested individually in triplicate 1.5 L aquaria each of which contained 10 larvae. All aquaria were provided with gentle aeration and kept in a heated room (constant air temperature of 29±1 °C), maintaining the water temperature at 26±1 °C. Filtered synthetic freshwater was used as fish culture water. To maintain the NH_4^+ and NO_2^- levels below 0.5 mg

NH_4^+ $L^{-1}$and 0.2 mg NO_2^- L^{-1}, respectively, 25 % of the culture water was renewed every 3 d during the 12 d experimental period.

Fish were challenged daily over the experimental period using 3 challenge methods: (1) via culture water, (2) via axenic *Artemia* nauplii, and (3) via both culture water and axenic *Artemia* nauplii. For the culture water challenges, bacterial suspensions were harvested by centrifuging at 1000 × *g* for 10 min and washed twice in their respective culture medium followed by 1 time washing using fish culture water before addition. The density of the bacterial suspension prior to addition was determined based on the McFarland standard (BioMérieux, Marcy L'Etoile, France) by measuring the turbidity with a spectrophotometer (Genesys 20, Thermospectronic) at 550 nm. For each replicate tank, bacteria were added at a density of 10^6 CFU mL^{-1}.

For the challenge via axenic *Artemia* nauplii, axenic *Artemia* cysts were incubated and hatched following the procedure of Marques et al. (2004). Axenic *Artemia* nauplii were harvested after 24 h incubation at 27±1 °C, washed using filtered autoclaved synthetic freshwater, and counted. Bacterial suspensions were harvested by centrifuging at 1000 × *g* for 10 min and washed twice using their respective culture medium and added to the axenic *Artemia* culture (100 *Artemia* mL^{-1}) at a density of 10^8 CFU mL^{-1}. Bacteria loaded *Artemia* nauplii were harvested after 1 h incubation and washed twice using sterile saline solution (9 g L^{-1} NaCl), counted, and added into the tanks at the density of 20 *Artemia* $fish^{-1}$. For the challenge via both *Artemia* nauplii and culture water, both challenges were conducted as described above. For all challenge methods, mortality observation and dead fish removal were done twice daily. The bacterial load of the *Artemia* nauplii in each treatment was determined by the plate count method. After rinsing and counting, subsamples of bacteria loaded *Artemia* were homogenized according to the procedure described by Huys et al. (2001). Subsequently, 50 µL of the homogenized *Artemia* suspension was dilution plated (Spiral plater™, Spiral

Systems, USA) on LB agar. The inoculated plates were incubated overnight at 27±1 °C and the grown colonies were counted.

Development of challenge test on gnotobiotic Nile tilapia larvae

Disinfection protocol to obtain axenic Nile tilapia larvae

Nile tilapia were naturally bred in our laboratory and 3 d post fertilization (3 dpf) eggs were collected and pooled following the procedures described above for the xenic pathogen-screening tests. Upon collection, eggs were put on a sterile nylon sieve (mesh size 300 μm) and washed 4 times using 250 mL 0.2 μm-filtered autoclaved synthetic freshwater at 25±1 °C to remove loose bacteria. Unfertilized eggs, dead eggs or eggs with ruptured yolk were discarded prior to the disinfection procedure. A double disinfection procedure was applied for the remaining eggs at the eyed egg stage (stage 14-15) (Fujimura and Okada, 2007). In the first disinfection step, eggs were immersed in diluted 30 % hydrogen peroxide (MERCK-Schuchardt 386790) with a final active peroxide concentration of 2000 mg L^{-1} for 10 min at 25±1 °C. During disinfection, eggs were gently agitated to ensure that all eggs had equal contact with the disinfecting agent. Subsequently, the eggs were rinsed 4 times using 250 mL 0.2 μm-filtered autoclaved synthetic freshwater. Next, the eggs were incubated in 0.2 μm-filtered autoclaved synthetic freshwater containing 10 mg L^{-1} each of ampicillin (Sigma-A0166), rifampicin (Sigma-83907), trimethoprim (Sigma-T7883) and gentamycin (Sigma-G1264) and the antifungal agents Amphotericin-B (Sigma-A9528) and Fluorescent Brightener 28 (Sigma-F3543) at concentrations of 0.5 mg L^{-1} and 25 mg L^{-1}, respectively. The second disinfection step was done 24 h following the first disinfection. Eggs were first rinsed from the culture medium from the first disinfection using 500 mL 0.2 μm-filtered autoclaved synthetic freshwater. A sodium hypochlorite solution with 75 mg L^{-1} active chlorine (Sigma Aldrich 425044) was prepared in 0.2 μm-filtered autoclaved synthetic freshwater. Eggs were then immersed in this solution for 2 min at 25±1 °C. Following disinfection, eggs were rinsed 4 times using 250 mL 0.2

μm-filtered autoclaved synthetic freshwater. All disinfection procedures were performed in a laminar flow hood.

Disinfected eggs were aseptically distributed to 500 mL sterile glass bottles containing 200 mL 0.2 μm-filtered autoclaved synthetic freshwater at a density of 500 eggs L^{-1}. Eggs of the control group underwent the same incubation procedure, however, they were not surface disinfected or treated with antibiotic and antifungal agents. Each incubation bottle was equipped with 2 sterile 0.2 μm filters (Sartorius Midisart®, USA) for the aeration in- and outlet to provide gentle sterile aeration during incubation. Eggs hatched after 3 days of incubation following the disinfection procedure and larvae were maintained in the axenic incubation medium throughout the non-feeding larval stage. The effect of the disinfection procedure on the egg hatching success was evaluated using the egg hatching percentage, which was measured as the proportion of the hatched eggs relative to the total eggs incubated regardless of the viability (Komara et al., 2004). The hatching percentage was determined 1 DAH.

Tests for axenity

Axenity was checked at several crucial steps during the experiments. After 24 h following the disinfection procedure, 5 eggs were aseptically sampled from each incubation bottle, individually homogenized and plated on Lysogeny broth (LB) medium + 15 g L^{-1} agar (LB Agar; Bacteriological Grade, MP Biomedicals). In addition, 1 mL of water from each incubation bottle was added into a sterile tube containing 9 mL of LB broth (10 %). The inoculated tubes were incubated at 27±1 °C for 96 h and checked for any bacterial / fungal growth.

At stocking of the larvae (6 DAH) and at the end of the gnotobiotic challenge test, the fish larvae from the axenic treatments were checked for bacterial contamination using the plate culture method. From each incubation bottle, 2 larvae were euthanized using 1 g L^{1} sterile benzocaine and washed with 1 g L^{-1} sterile benzalkonium chloride before being rinsed with 0.2 μm-filtered autoclaved

synthetic freshwater and homogenized in sterile saline solution (9 g L^{-1} NaCl). Subsequently, 50 µL of the homogenate was plated (Spiral plater™, Spiral Systems, USA) on LB agar plates. The inoculated plates were incubated at 27±1 °C for 96 h and checked for any bacterial / fungal growth.

Bacterial challenge procedure on gnotobiotic Nile tilapia larvae

Along with the antifungal agents (0.5 mg L^{-1} Amphotericin-B and 25 mg L^{-1} Fluorescent Brightener-28), a mixture of antibiotics containing ampicillin, rifampicin, kanamycin, trimethoprim and gentamycin (each at 10 mg L^{-1}) was used in the larval incubation medium throughout the gnotobiotic challenge tests. Therefore, it was necessary to identify antibiotic resistant mutants of the pathogens that could be used for challenge. The resistance of *E. ictaluri* gly09 to the different antibiotics was investigated by inoculating an overnight *E. ictaluri* gly09 culture into sterile tubes containing BHI broth and one of the 5 antibiotics at a concentration of 10 mg L^{-1}. These cultures were incubated overnight on a horizontal shaker at 160 rpm at 27±1 °C. Growth, determined by the increase in turbidity, was obtained in the presence of 10 mg L^{-1} ampicillin, trimethoprim and gentamycin, suggesting that *E. ictaluri* gly09 is intrinsically resistant to these antibiotics. No growth occurred in the presence of 10 mg L^{-1} rifampicin or kanamycin.

In order to obtain spontaneous rifampicin-resistant and kanamycin-resistant *E. ictaluri* gly09 mutants, the wild type *E. ictaluri* gly09 strain was cultured separately on BHI agar plates containing 2 mg L^{-1} of each antibiotic. Colonies appearing on the BHI-rifampicin and BHI-kanamycin plates after an incubation period ranging from 48 to 72 h at 27±1 °C were harvested, mixed and transferred into several tubes containing BHI broth with 2 mg L^{-1} of both antibiotics. Cultures were incubated on a horizontal shaker at 160 rpm at 27±1 °C overnight, after which 1 mL of culture was transferred into new tubes containing 9 mL of BHI broth with a higher antibiotics concentration of 5 mg L^{-1} each. Following overnight incubation, grown cultures (cultures with an absorbance level of more than 0.5 at a wavelength of 600 nm) were then transferred into new tubes containing 9 mL of BHI broth with

higher antibiotics concentration of 10 mg L^{-1}. Cultures of *E. ictaluri* gly09 that were resistant to 10 mg L^{-1} rifampicin and kanamycin were transferred and grown in BHI broth containing 10 mg L^{-1} ampicillin, rifampicin, kanamycin, trimethoprim and gentamycin. The resulting *E. ictaluri* gly09 strain (referred to as *E. ictaluri* gly09R) with multiple antibiotics resistance was used in the gnotobiotic challenge tests. *E. ictaluri* gly09R culture was washed from its culture medium prior to enrichment of *Artemia* nauplii; thus, no nutrient was provided to the bacteria during the challenge test. Axenic fish larvae were challenged with *E. ictaluri* gly09R via both axenic *Artemia* nauplii and culture water following the procedures applied in the pathogen-screening test on xenic larvae. The *Artemia* nauplii were considered as the only nutritional sources for the fish larvae.

The gnotobiotic challenge tests were done in triplicate in 500 mL sterile glass bottles containing 200 mL 0.2 μm-filtered autoclaved synthetic freshwater with 10 fish per bottle. Each incubation bottle was equipped with 2 sterile 0.2 μm air filters (Sartorius Midisart®, USA) to provide gentle sterile aeration and a sterile septum for aseptic larval feeding during the experimental period. An unchallenged axenic fish group was used as the control group for the gnotobiotic system. In order to verify that there was no effect of the culture system set-up on the pathogen virulence, in experiment 2a the *E. ictaluri* gly09R strain was also tested on xenic larvae using the same culture system set-up but without the 0.2 μm air filtration or the use of both antibiotics and antifungal mixtures. To maintain good environmental conditions within the experimental system, 25 % of the incubation medium with or without antimicrobials was replaced every 3 days. The gnotobiotic challenge test was repeated twice (experiment 2a and 2b). In experiment 2a, *E. ictaluri* gly09R strain was tested on both xenic and gnotobiotic fish, while in experiment 2b it was only tested on gnotobiotic fish.

In order to determine larval bacterial load at the end of the challenge test, surviving larvae were killed using an overdose of benzocaine, surface-disinfected in a bath of 1 g L^{-1} benzalkonium chloride and rinsed with 0.2 μm-filtered autoclaved synthetic

freshwater, then homogenized and plated on BHI agar containing 10 mg L^{-1} ampicillin, rifampicin, kanamycin, trimethoprim and gentamycin, according to the procedure described earlier. The inoculated plates were incubated overnight at 27±1 °C and the grown colonies were counted.

Bacterial challenge test for gnotobiotic Nile tilapia larvae supplied with poly-β-hydroxybutyrate (PHB)

The developed bacterial challenge procedure on gnotobiotic Nile tilapia larvae was further employed to evaluate the effect of PHB on the survival of challenged larvae. In this experiment (experiment 3), 160 axenic larvae (DAH 5) were distributed in 4 treatment groups with four replicates: (1) control group - no PHB supplementation and unchallenged, (2) PHB-enriched and unchallenged group, (3) PHB-enriched and challenged group, and (4) challenged group without PHB enrichment. The bacterial challenge was done daily during the 12 days experimental period via the culture water and axenic *Artemia* nauplii. Bacterial challenge via water culture and via *Artemia* nauplii was done following the procedures applied in the previous challenge tests.

For the fish groups supplied of PHB, *Artemia* nauplii at a density of 100 *Artemia* mL^{-1} were enriched in a 100 mg L^{-1} PHB suspension for 1 h, washed with 0.2 μm-filtered autoclaved synthetic freshwater and added to its respective incubation bottles. The feeding level (i. e., the total number of *Artemia* nauplii consisting of non-enriched, pathogen-enriched, and/or PHB-enriched given to each replicate bottle) was the same for all treatments. The control group fish were fed with axenic non-enriched *Artemia* nauplii at the density of 20 nauplii per fish. Unchallenged or challenged PHB-supplemented fish were fed PHB-enriched *Artemia* nauplii at the density of 10 nauplii per fish with an addition of axenic *Artemia* nauplii or pathogen-enriched *Artemia* nauplii, respectively, at a density of 10 nauplii per fish. The challenged fish without PHB supplementation were fed axenic *Artemia* nauplii and pathogen-enriched *Artemia* nauplii, each at the density of 10 nauplii per fish. Mortality observation and dead fish removal was done daily during the

experiment. The experimental designs for bacterial challenge tests on Nile tilapia larvae were approved by the ethical committee of Ghent University under the file number EC2012/070 for experiment 1a and 1b, EC2013/069 for experiment 2a and 2b, and EC2014/041 for experiment 3.

Statistical analysis

A chi-square test was used to detect significant differences in the hatching percentage between the xenic and the disinfected eggs. For the comparison of the cumulative mortality of fish larvae, data were arcsine transformed before a one-way analysis of variance (one-way ANOVA) was performed using STATISTICA statistical software (version 7.0). A Duncan test was performed on the transformed data for multiple comparisons among means. All analyses were run at a minimum level of significance of 5 %. The relative percentage of survival (RPS), which is the larval survival after challenge when compared to control fishes (Amend, 1981), was calculated to evaluate the efficacy of xenic and gnotobiotic bacterial challenge test as follows:

$$\text{RPS (\%)} = \left[1 - \left(\frac{\text{\% mortality in challenge group}}{\text{\% mortality in control group}}\right)\right] \times 100$$

The RPS obtained in the challenge studies were analysed using a chi-square test.

Results

Pathogen-screening tests on xenic Nile tilapia larvae

In the first pathogen-screening test (experiment 1a), mortality was first observed in the group challenged with *E. ictaluri* gly09 via *Artemia* nauplii and in the group challenged with *E. ictaluri* gly10 via the culture water 6 days after challenge (6 DAC) (Table 5.1). There were no significant differences between treatments (challenge routes and control) until 11 DAC when significantly higher levels of mortality were seen in the groups challenged with *E. ictaluri* gly09 via *Artemia* and via both *Artemia* and culture water when compared to the control and the groups

challenged via the culture water. A similar result was observed for *E. ictaluri* gly10 with the exception that the group challenged via *Artemia* had significantly higher mortalities than all other treatment groups and the control at 10 DAC. At 11 DAC, significantly higher levels of mortality were observed in the groups challenged via *Artemia* and via both *Artemia* and culture water when compared to the control and other groups challenged via culture water (Table 5.1).

No significant difference in fish mortality was observed between the treatment groups challenged with *S. agalactiae* LMG15977 and the control group. Furthermore, significantly lower mortalities were observed when compared the groups challenged with *S. agalactiae* LMG15977 to the groups challenged with *E. ictaluri* strains via *Artemia* and via both *Artemia* and culture water (Table 5.1). The pathogenicity of both *E. ictaluri* strains for tilapia larvae was confirmed in experiment 1b (Table 2), where the treatment groups challenged with either *E. ictaluri* gly09 or gly10 via *Artemia* and culture water resulted in significant mortalities of 93±11 % and 100±0 %, respectively, as compared to the 40±17 % mortality seen in the control group at 9 DAC. A significantly higher larval mortality was observed in the group challenged with *E. tarda* LMG2793 via *Artemia* and culture water when compared to the control group, starting from 7 DAC with a final mortality of 93±11 % at 9 DAC (Table 5.2).

Table 5.1. Cumulative mortality (%, Mean±SD) of control and challenged (*E. ictaluri* gly09, *E. ictaluri* gly10 and *S. agalactiae* LMG15977) xenic Nile tilapia larvae using different challenge methods in experiment 1a. Different letters within the same row denote significant differences ($P \leq 0.05$) ($n = 3$; m=10). n = number of replicates; m = initial number of fish larvae per replicate. DAC = day after challenge.

		Edwardsiella ictaluri gly09			*Edwardsiella ictaluri* gly10			*Streptococcus agalactiae* LMG15977		
	Control	via culture water	via *Artemia* nauplii	via *Artemia* and water	via culture water	via *Artemia* nauplii	via *Artemia* and water	via culture water	via *Artemia* nauplii	via *Artemia* and water
6 DAC	0±0^{a}	0±0^{a}	4±6^{a}	0±0^{a}	4±6^{a}	0±0^{a}	0±0^{a}	0±0^{a}	0±0^{a}	0±0^{a}
7 DAC	0±0^{a}	4±6^{a}	7±6^{a}	0±0^{a}	4±6^{a}	4±6^{a}	0±0^{a}	0±0^{a}	0±0^{a}	4±6^{a}
8 DAC	0±0^{a}	33±29^{a}	30±17^{a}	18±6^{a}	18±13^{a}	41±28^{a}	33±29^{a}	18±23^{a}	4±6^{a}	15±17^{a}
9 DAC	26±26^{a}	44±22^{a}	56±11^{a}	52±6^{a}	41±6^{a}	63±28^{a}	74±6^{a}	22±29^{a}	26±28^{a}	37±26^{a}
10 DAC	37±23^{a}	59±6^{a}	74±13^{a}	81±6^{a}	48±6^{a}	74±17^{a}	85±6^{b}	44±22^{a}	48±28^{a}	63±17^{a}
11 DAC	48±13^{a}	67±0 ^{a}	81±6^{b}	81±6^{b}	70±6^{a}	81±13^{b}	89±0^{b}	67±19^{a}	70±13^{a}	74±6^{a}

Table 5.2. Cumulative mortality (%, Mean±SD) of control and challenged (*E. ictaluri* gly09, *E. ictaluri* gly10 and *E. tarda* LMG2793) xenic Nile tilapia larvae via both *Artemia* nauplii and culture water in experiment 1b. Different letters within the same row denote significant differences ($P \leq 0.05$) ($n = 3$; m = 10). n = number of replicates; m = initial number of fish larvae per replicate. DAC = day after challenge.

	Control	*E. ictaluri* gly09	*E. ictaluri* gly10	*E. tarda* LMG2793
5 DAC	0±0^{a}	3±6^{a}	0±0^{a}	0±0^{a}
6 DAC	0±0^{a}	17±6^{a}	17±21^{a}	3±6^{a}
7 DAC	0±0^{a}	37±31^{ab}	47±25^{b}	60±17^{b}
8 DAC	27±23^{a}	77±6^{b}	100±0^{c}	83±15^{b}
9 DAC	40±17^{a}	93±11^{b}	100±0^{b}	93±11^{b}

Eggs disinfection protocol for axenic Nile tilapia larvae

The effect of the disinfection procedure on the egg hatching success was evaluated by measuring the hatching percentage at 1 DAH for the xenic and the disinfected eggs. There was no significant effect of the disinfection procedure on the hatch percentage in both gnotobiotic experiments (Fig. 5.1). With respect to the egg / larvae axenity following the egg disinfection procedure, samples of the homogenized eggs that were taken 24 h after disinfection never resulted in growth on the LB plates after 96 h incubation. Samples of the incubation medium or homogenized larvae at larval stocking also did not show any colonies on the LB plates after 96 h incubation. In the course of the experiment, no bacteria were detected in the samples of the axenic fish at the end of experiment 2a, as verified by fish homogenate plating on the LB plates. A very low level of bacterial contamination was observed in the axenic group at the end of experiment 2b, as bacteria colonies grew on the LB plates after 96 h incubation at a density of <30 CFU fish^{-1}.

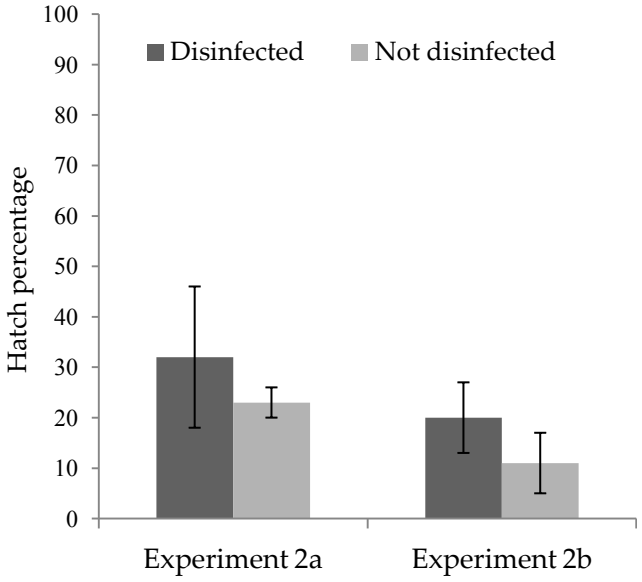


Figure 5.1. Hatch percentage of the disinfected and xenic (not disinfected) eggs in gnotobiotic experiments. Bars represent the mean values of three treatment replicates; error bars represent standard deviation values. No significant difference was observed between the disinfected and not disinfected eggs in both experiments ($P > 0.05$).

Quantification of the bacteria load in Artemia nauplii and Nile tilapia larvae

In experiment 1a, incubation of axenic *Artemia* nauplii with *E. ictaluri* gly09, *E. ictaluri* gly10 and *S. agalactiae* LMG15977 resulted in a density of 7.8±0.2, 5.2±0.1, and 6.5±0.1 × 10^2 CFU *Artemia*$^{-1}$ after 1 h of incubation, respectively. Similar results were observed in experiment 1b, where incubation of axenic *Artemia* nauplii resulted in a density of 6.9±0.7, 5.1±0.4, and 7.7±0.2 × 10^2 CFU *Artemia*$^{-1}$ for *E. ictaluri* gly09, *E. ictaluri* gly10 and *E. tarda* LMG2793, respectively.

In the challenge tests using the gnotobiotic system, the *E. ictaluri* gly09R loaded onto the *Artemia* nauplii resulted in a density of 4.5±0.9 × 10^3 CFU *Artemia*$^{-1}$ and 8.8±0.5 × 10^2 CFU *Artemia*$^{-1}$ after 1 h incubation in experiment 2a and 2b, respectively. At the end of the gnotobiotic challenge test, the density of *E. ictaluri* gly09R inside the surviving fish was 7.9±0.9 × 10^4 CFU fish^{-1} and 4.8±0.6 × 10^3 CFU fish^{-1} in experiment 2a and 2b, respectively.

Mortality of the gnotobiotically challenged Nile tilapia larvae

In both gnotobiotic challenge tests, the mortality of the fish after challenge by *E. ictaluri* gly09R strain was not significantly different ($P > 0.05$) compared to the unchallenged (axenic or xenic) fish until 9 DAC (Table 5.3). From 10 DAC onwards, the challenge of the axenic larvae with *E. ictaluri* gly09R resulted in a significantly higher mortality ($P \leq 0.05$) as compared to the unchallenged axenic fish in both challenge tests. Challenge with the *E. ictaluri* gly09R strain under xenic conditions resulted in a significant mortality ($P \leq 0.05$) of 40±10 % as compared to the xenic unchallenged group, but no significant differences were observed when compared to the gnotobiotically challenged group (12 DAC; experiment 2a). With respect to the RPS, there was no significant difference in mortality ($P > 0.05$) between the axenic fish challenged with *E. ictaluri* gly09R and the xenic fish challenged with *E. ictaluri* gly09R at 12 DAC, with a RPS of 67 and 73 %, respectively.

Effect of PHB in a bacterial challenge test on gnotobiotic Nile tilapia larvae

In experiment 3, the enrichment of *Artemia* nauplii with pathogen *E. ictaluri* gly09R resulted in a bacterial load of 3.1 x 10^3 CFU Artemia^{-1}. Larval mortality was first observed in all treatments on 6 DAC (Table 5.4). While a low level of mortality was observed in the unchallenged groups, the mortality level was significantly increased in the challenged groups. At the end of the experiment (11 DAC), the challenged larve without PHB supplementation showed the highest mortality level of 70±8 %, which was significantly higher than the challenged fish with PHB supplementation (50±8 %) and the unchallenged fish with or without PHB supplementation (20±8 % and 28±10 %, respectively).

Table 5.3. Cumulative mortality (%, Mean±SD) of xenic (not challenged or challenged), axenic and gnotobiotically challenged Nile tilapia larvae using antibiotics-resistant *E. ictaluri* gly09R strain via *Artemia* nauplii and culture water in experiment 2a and 2b. Different letters within the same row within 1 experiment denote significant differences ($P \leq 0.05$) (n = 3; m = 10). n = number of replicates; m = initial number of fish larvae per replicate. DAC = day after challenge.

	Experiment 2a				Experiment 2b	
	Xenic	Xenic *E. ictaluri* gly09R	Axenic	Gnotobiotic *E. ictaluri* gly09R	Axenic	Gnotobiotic *E. ictaluri* gly09R
5 DAC	0±0^{a}	3±6^{a}	0±0^{a}	0±0^{a}	0±0^{a}	0±0^{a}
6 DAC	3±6^{a}	3±6^{a}	0±0^{a}	0±0^{a}	0±0^{a}	0±0^{a}
7 DAC	7±6^{a}	3±6^{a}	0±0^{a}	0±0^{a}	0±0^{a}	0±0^{a}
8 DAC	7±6^{a}	10±10^{a}	0±0^{a}	0±0^{a}	7±6^{a}	10±10^{a}
9 DAC	7±6^{a}	10±10^{a}	0±0^{a}	3±6^{a}	13±6^{a}	23±11^{a}
10 DAC	7±6^{ab}	13±6^{b}	0±0^{a}	13±6^{b}	20±0^{a}	53±15^{b}
11 DAC	7±6^{a}	23±6^{ab}	10±10^{ab}	33±15^{b}	20±0^{a}	60±10^{b}
12 DAC	13±6^{a}	40±10^{bc}	23±6^{ab}	57±6^{c}	23±6^{a}	63±11^{b}

Table 5.4. Cumulative mortality (%, Mean±SD) of gnotobiotically challenged Nile tilapia larvae using antibiotics-resistant *E. ictaluri* gly09R strain via *Artemia* nauplii and culture water in experiment 3. Different letters within the same row denote significant differences ($P \leq 0.05$) (n = 4; m=10). n = number of replicates; m = initial number of fish larvae per replicate. DAC = day after challenge.

	Axenic	PHB-enriched	Challenged with *E. ictaluri* gly09R	PHB-enriched and challenged with *E. ictaluri* gly09R
6 DAC	3±5[a]	3±5[a]	3±5[a]	3±5[a]
7 DAC	3±5[a]	3±5[a]	10±8[a]	3±5[a]
8 DAC	8±5[a]	3±5[a]	13±13[a]	5±6[a]
9 DAC	10±0[a]	3±5[a]	30±18[b]	10±8[a]
10 DAC	20±12[a]	18±10[a]	60±8[c]	40±12[b]
11 DAC	28±10[a]	20±8[a]	70±8[c]	50±8[b]

Discussion

Pathogen-screening tests on xenic Nile tilapia larvae

Streptococcus sp. and *Edwardsiella* sp. are common genera of bacteria that have been reported to infect wild and farmed tilapia (Plumb and Hanson, 2010). *E. tarda* has been reported to infect and cause significant pathology in red tilapia (Iregui et al., 2012). In the last few years, different strains of *E. ictaluri*, which are known as etiological agents of ESC, have also been reported as the causative agent of morbidity and mortality in Nile tilapia fingerlings (Soto et al., 2012). In this study, four candidate pathogenic bacterial strains representing the two groups were screened for the development of a bacterial challenge test for gnotobiotic Nile tilapia larvae: *E. ictaluri* gly09, *E. ictaluri* gly10, *E. tarda* LMG2793 and *S. agalactiae* LMG15977.

The bacterial strain *S. agalactiae* LMG15977, which was the only bacterial strain actually isolated from tilapia culture, resulted in no significant mortality in tilapia larvae. Several studies suggested that the susceptibility of Nile tilapia to *S. agalactiae* infection is temperature dependent (Mereghetti et al., 2008; Rodkhum et al., 2011). It is possible that the pathogenicity of *S. agalactiae* in this experiment was affected by the water temperature that was below the optimal growth temperature for *S. agalactiae* culture.

Unlike *S. agalactiae*, both *E. ictaluri* strains can cause significant larval mortalities when associated with the larval feed *Artemia*, with or without additional pathogen delivery via the culture water. However, less or non-significant mortality was observed when the fish were only exposed to the pathogens via the culture water. In a study by Soto et al. (2012), tilapia fingerlings (~15 g) challenged with *E. ictaluri* at 10^6 CFU mL^{-1} showed 100 % mortality at 8 d post immersion. The results of the current study suggest that bacterial infection via culture water in tilapia larvae (immersion challenge) is less likely than infection through feed uptake.

Due to the emergent nature of edwardsiellosis in non-ictalurid fish, little is known regarding the dynamics of *E. ictaluri* infection in Nile tilapia culture, especially during its larviculture. To our knowledge, this is the first report of *E. ictaluri* induced mortality in Nile tilapia larviculture. With respect to the study by Soto et al. (2013) these authors increased our understanding on the pathogenesis of *E. ictaluri* in Nile tilapia fingerlings suggesting that the cutaneous and oral routes were the main ports of entry for the bacterium, which then spreads hematogenously throughout the fish body, with the spleen and head kidney as the main targets of infection. Their observations support the study by Li et al. (2012), who suggested that *E. ictaluri* gains entry through the intestinal epithelium, possibly using actin polymerization and receptor-mediated endocytosis as mechanisms of invasion.

In both of the pathogen screening tests, the first fish mortalities in the treatment group challenged with *E. ictaluri* occurred at 5–6 DAC. However, the mortality from the challenges via both *Artemia* and culture water in experiment 1a differed when compared to experiment 1b as significant differences in mortality following challenge occurred as already at 7 DAC. The differences in the incubation period until significant mortalities were observed could be caused by the presence of other microorganisms beside the tested pathogens or a difference in pathogen resistance between the different larval batches.

The results of both pathogen-screening tests showed that all *Edwardsiella* strains tested in this study can cause significant mortality in tilapia larvae culture and are candidates for use in the development of a bacterial challenge test on gnotobiotic Nile tilapia larvae, with the challenge route via *Artemia* nauplii, with or without additional challenge via the culture water, as an effective procedure.

Eggs disinfection protocol for axenic Nile tilapia larvae

Compared to the hatch percentage of tilapia eggs that are commonly incubated in upwelling fish egg incubators (Rana, 1988), the relatively low hatch percentage

obtained in this study could be due to the type of incubation system required to maintain the axenic conditions that might not be optimal for egg hatching incubation. In order to obtain axenic larvae, artificial incubation of surface-disinfected eggs in axenic conditions until hatching is crucial. These included the need for a gentle motion of the eggs and the need for an axenic closed system with easy access for removal of non-developing embryos and also refreshment of sterile culture medium.

In gnotobiotic studies with fish, the common method to obtain axenic larvae is to collect fertilized eggs, disinfect them and then incubate them in a cocktail of disinfectants and antibiotics. The effectiveness of sodium hypochlorite and hydrogen peroxide solutions as egg surface disinfectant has been reported by several studies with different fish species using various concentrations and exposure times (Barnes et al., 1998; Douillet and Holt, 1994; Marking et al., 1994; Mitchell et al., 2009; Pham et al., 2008; Rach et al., 1998; Schreier et al., 1996). In this study, the results of the axenity test suggest that the double disinfection using hydrogen peroxide (2000 mg L^{-1} for 10 min) and sodium hypochlorite (75 mg active chlorine L^{-1} for 2 min), followed by incubation in a medium containing antibiotics (10 mg L^{-1} ampicillin, rifampicin, trimethoprim, and gentamycin) and antifungal agents (0.5 mg L^{-1} Amphotericin-B and 25 mg L^{-1} Fluorescent Brightener 28) was effective to obtain axenic Nile tilapia larvae. To our knowledge, this is the first published protocol to generate gnotobiotic Nile tilapia larvae. The low bacterial contamination observed at the end of experiment 2b could be related to the technical complexity of the system (Marques et al., 2006), where handlings and manipulations, such as the culture medium exchange and daily feeding involved during the experimental period might have resulted into contamination.

Mortality of the gnotobiotically challenged Nile tilapia larvae

This laboratory test system relied on antibiotics to maintain gnotobiotic conditions. As for this, antibiotic resistant mutants need to be isolated / developed prior to use in challenge trials. Such spontaneous mutations have been found in several Gram-

negative bacteria, including *Edwardsiella* species (Ingham and Furneaux, 2000; Thavasi et al., 2007). It was reported that *E. ictaluri* strains are naturally resistant to rifampicin, macrolides, lincosamides, streptogramins, glycopeptides, fusidic acid, oxacillin (Stock and Wiedemann, 2001) and gentamycin (Reger et al., 1993). Several studies also reported *E. ictaluri* isolates displaying acquired resistance to trimethoprim, streptomycin, oxytetracycline (Dung et al., 2008), kanamycin (Russo et al., 2009), and ampicillin (Russo, 2011). In this study, the multiple antibiotic resistance of *E. ictaluri* gly09R was both intrinsic (for ampicillin, trimethoprim and gentamycin) and mutational in nature (for rifampicin and kanamycin).

When creating antibiotic resistance in a bacterium, either via plasmids or by spontaneous mutation, the acquired resistance represents an additional energy cost for the strain that can result in a longer generation time and altered metabolic activity (Andersson and Levin, 1999; Feng and Rise, 2009; Levin et al., 2000; McDermott et al., 2006). This implies that a bacterial strain with introduced resistance may behave differentially from the original strain. The *E. ictaluri* gly09R strain used in this study, however, showed similar pathogenicity in the gnotobiotically challenged fish as compared to the xenic challenged fish, indicating that the bacterial pathogenicity was preserved after the acquisition of antibiotic resistance.

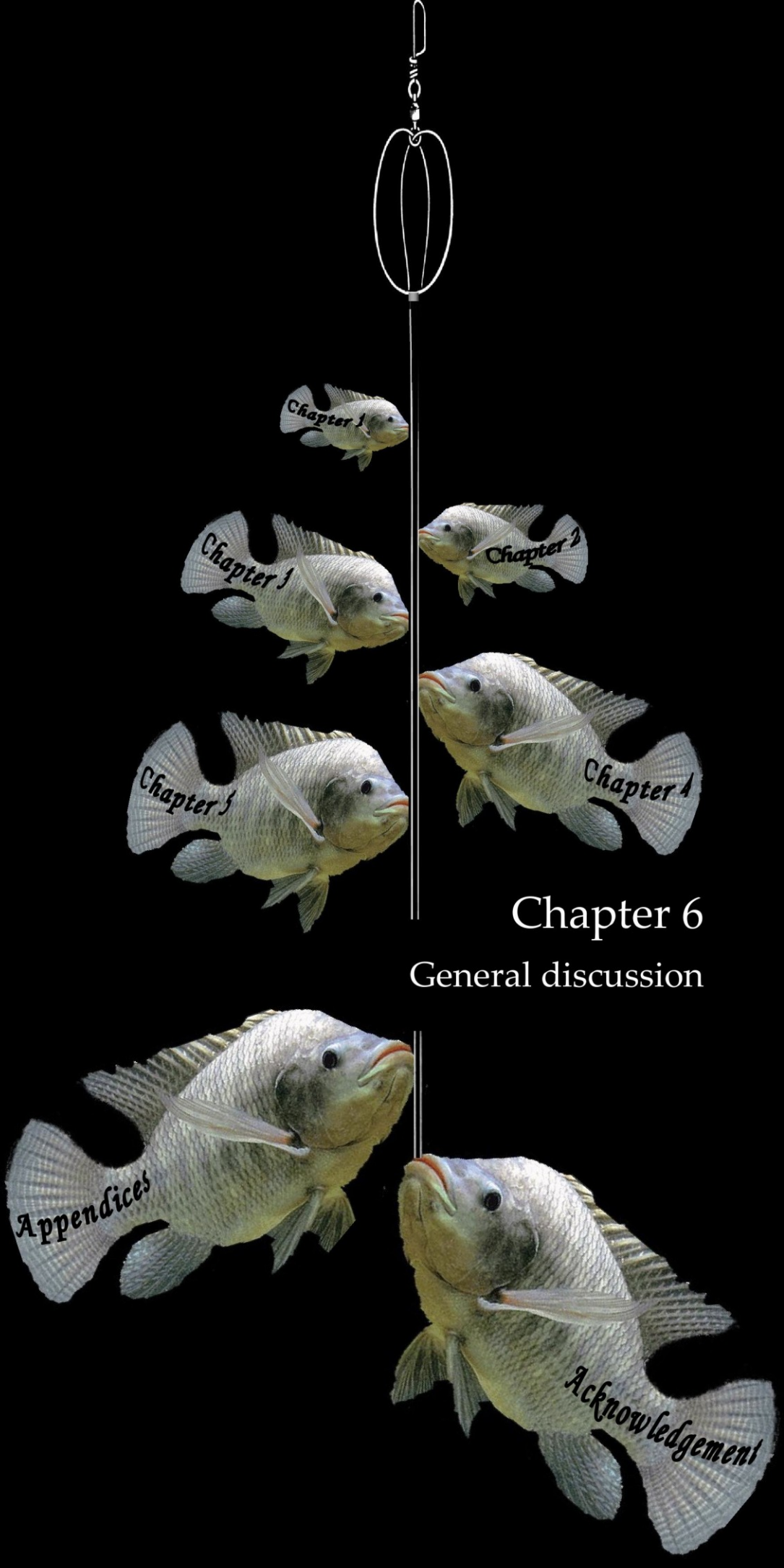
Several studies on gnotobiotic culture systems suggested that the cellular and humoral defence systems of axenic animals is underdeveloped hence making them more susceptible to diseases when compared to xenic animals (Coates, 1975; Marques et al., 2006). Unlike these previous studies, the similar RPS values between the gnotobiotic and xenic fish in the gnotobiotic challenge test did not indicate that axenic Nile tilapia larvae are more susceptible to pathogen infection compared to xenic larvae. The bacterial challenge in a gnotobiotic environment thus yields similar results as in a conventional open system, however without uncontrolled interference.

Effect of PHB in a bacterial challenge test on gnotobiotic Nile tilapia larvae

The gnotobiotic challenge test was used to investigate if PHB has a beneficial effect by protecting the tilapia larvae against *E. ictaluri* gly09R infection. Similarly to the study in prawn larviculture (Thai et al., 2014), this experiment showed that PHB supplementation to gnotobiotic Nile tilapia larvae can provide certain protection against pathogenic infection. Despite the evidence of the beneficial effects of PHB in disease protection, knowledge on the actual protective mechanism(s) of PHB remains scarce. It will be of interest to further evaluate the effect of PHB on the growth and pathogenicity of *E. ictaluri* gly09R culture. In addition, the availability of the gnotobiotic Nile tilapia challenge test creates the opportunity for a detailed investigation of the effects of PHB exposure on pathogen growth and virulence under microbiologically controlled conditions in Nile tilapia.

Conclusions

This study succeeded in developing a Nile tilapia gnotobiotic challenge system using *E. ictaluri* gly09R as a pathogen. The usefulness of this model was shown for PHB which upon application through PHB-loaded *Artemia*, decreased the susceptibility of the larvae to a pathogenic challenge. The gnotobiotic system will be a useful tool to extend the understanding of the mechanisms involved in host–microbe interactions and will as such contribute to the development of biocontrol strategies to be applied in aquaculture practices.



Chapter 6

General discussion

Chapter 6

General discussion and conclusions

The routine use of chemical compounds and antibiotics as growth promoters and conventional agent of disease control is a matter of debate in the animal farming industry. The development of non-antibiotic and environment friendly agents is one of the key targets for health management in aquaculture. Consequently, with the emerging need for environment friendly aquaculture, the use of alternatives to antibiotic growth promoters in fish nutrition is now widely accepted. From the findings of this study, it is clear that the application of poly-β-hydroxybutyrate (PHB) works beneficially for growth and health promotion in Nile tilapia culture. The exact mechanisms of action of PHB, however, require further thorough exploration in order to optimally apply PHB at full-scale. In this chapter, the results obtained in this PhD research are integrated with the literature and discussed in the light of research needs and practice opportunities.

Evaluation of PHB as a growth promoter in aquaculture

Effects of dietary PHB on the fish growth

Many studies have explored the potential of PHB for aquaculture purposes by focusing on the beneficial effects at the level of the host. Different aquatic animals including *Artemia*, giant black tiger shrimp, giant freshwater prawn, Chinese mitten crab, European sea bass, and Siberian sturgeon have benefited from dietary PHB in terms of growth and health improvement (De Schryver et al., 2010; Defoirdt et al., 2007; Najdegerami et al., 2012; Nhan et al., 2010; Sui et al., 2012; Thai et al., 2014). For Nile tilapia culture, it was not yet assessed if dietary PHB has any impact on fish performance. It was therefore of importance that in the first step in this research it was verified that PHB showed no adverse and preferably beneficial growth effects on cultured Nile tilapia, which is a species of major importance in aquaculture. As expected, dietary PHB was found to improve the growth of Nile tilapia fingerlings (Chapter 3), although this growth promoting effect could not be detected in Nile tilapia juveniles (Chapter 2). Difference in growth between the two studies may be explained by the difference in age of the experimental fish. The first

study (Chapter 2) used tilapia juveniles at the age of 3-7 weeks old, while the second study (Chapter 3) used tilapia fingerlings at the age of 7-11 weeks old. Tengjaroenkul et al. (2000) reported that a considerable morphological change of the fish gut with complex coiling pattern occurs by the age of 9 weeks old, until the complexly-coiled definitive form as found in adult fish is obtained at 12 weeks old. The author also reported that the rate of increase in intestinal length takes place at an accelerating rate as the fish age – where the gut length increases from 90 % to 410 % of body length during the developmental period – and that the great intestinal length provides an advantage to the fish in digestion and absorption of nutrients. Thus, the difference in fish growth between the first and second study may be explained by the differences in development and performance of digestive system, which may have been resulted in different dietary nutrients (e.g. PHB) utilization capacities.

Based on this PhD study it can be suggested that the growth promoting effect of PHB is non-linear and varies for different developmental stages, because (1) a low PHB concentration of 1 g PHB kg^{-1} diet led to a similar growth of tilapia fingerlings as compare to a higher concentration of 50 g PHB kg^{-1} diet, and (2) a significant increase in growth was found in tilapia fingerlings but not in tilapia juveniles. This confirmed the result of a previous study on European sea bass, where dietary PHB supplementation at 20 g PHB kg^{-1} diet and 50 g PHB kg^{-1} diet resulted in a significant increase in growth of sea bass juveniles when compared to the control group. A higher concentration of 100 PHB kg^{-1} diet did, however, not lead to higher growth when compared to the lower PHB concentrations (20 g PHB kg^{-1} diet and 50 g PHB kg^{-1}) suggesting that the growth promoting effect of PHB is non-linear (De Schryver et al., 2010). Furthermore, in another study using Siberian sturgeon culture, dietary PHB supplementation at 20 g PHB kg^{-1} diet and 50 g PHB kg^{-1} diet led to similar growth of sturgeon fingerlings (Najdegerami et al., 2012), while the growth of sturgeon larvae when PHB supplemented with PHB through *Artemia* enrichment at 5 g L^{-1} (Najdegerami et al., 2013) led to reduced growth. In shrimp culture, Ludevese-Pascual et al. (personal communication) found no growth

stimulation of PHB on giant black tiger shrimp post-larvae. In another study, however, it was suggested that *Bacillus* sp. capable of accumulating PHB can provide beneficial effects on the growth performance of giant black tiger shrimp post-larvae (Laranja et al., 2014), although further investigations are required to verify the PHB effect of the bacterial cultures on the shrimp. Thus, based on the results of this current study and also the knowledge from the previous studies, it can be stated that the growth promoting effect of PHB is not only non-linear (dose-dependent), but also species-dependent and varies for different developmental stages. Hence, in order to apply certain PHB supplementation levels on targeted animal/species at specific age/developmental state, one should consider the differences in animal species (i.e. trophic level, feeding behaviour) and also developmental status. For each host species, it is worthwhile to first explore the optimal developmental stage at which PHB should/can be applied, as well as the optimal supplementation level.

Effects of dietary PHB on the fish lipid metabolism

Dietary proteins, lipids and carbohydrates are essential nutrients for growth (anabolism) and for energy to run the body machinery (catabolism) (Silva and Anderson, 1995). Lipids, especially phospholipids, are important for cellular structure and the maintenance of membrane flexibility and permeability (Sargent et al., 2002). Lipids as the primary energy providing nutrients in fish diets have a major protein-sparing effect in many fish species (Sargent et al., 2002; Wilson, 2002). In this study, the dietary PHB was found to significantly increase the whole-body total lipid content of Nile tilapia juveniles (Chapter 2). Increasing whole-body total lipid content was observed in Nile tilapia fingerlings, but it was not statistically different when compared to the control group (Chapter 3). Overall, based on the results of lipid analyses described in Chapter 2, it is hypothesized that the higher total whole-body lipid content in the PHB treatment groups may be due to larger amount of visceral fat, which is deposited in the form of fat (triglycerides).

It is suggested that changes in lipid content in fish body could be linked with changes in its synthesis and/or deposition rate in the muscle (Fauconneau 1984; Abdel-Tawwab et al. 2006). It is often reported that the muscle tissue of intensively cultured fish is characterized by increased lipid deposition consisting of mainly saturated fatty acid (SFA), monounsaturated fatty acid (MUFA) and linoleic acid (LA; 18:2n-6) (Karapanagiotidis et al., 2006). In this study, the increase in total lipid content of fish fed with PHB was also accompanied with an increase in the content of total SFAs, total MUFAs and total (n-6) FAs (Chapter 2). The composition of these FA groups (as proportion of total lipid content), however, remained unchanged. This indicates that fish FA profile was not affected by PHB supplementation. This is important as excessive deposition of LA is undesirable because it will reduce any conversion of alpha-linolenic acid (ALA; 18:3n-3) to eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), and will unbalance the production of eicosanoids toward the proinflammatory n-6 series prostaglandins in both the fish and the human consumer. However, several studies reported that fatty acid profiles in cultured fish are not only influenced by their dietary uptake and capacity to *de novo* synthesizes other fatty acids, but also by their capacity to bioconvert certain fatty acids (Anderson and Arthington, 1992). Maina et al. (2003) reported that tilapia possesses desaturation and elongation enzymes that efficiently convert C18 PUFAs to longer chain PUFAs, e.g. the capacity of bioconversion of LA (18:2 n-6) into ARA (20:4n-6) and LNA (18:3n-3) into EPA (20:5n-3). They also convert EPA into DPA (22:5n-3) and DPA into DHA (22:6n-3) (Nishiyama et al., 2014). Hence, in this study, the analyzed fish FA profile might reflect the FA profile of the experimental diets as well as the result of bioconversion of FA process by the fish.

The increase in whole-body total lipid content in the PHB treatment groups is also accompanied with the increase in lipase activity (Chapter 2). Similarly, the lipase activity of Siberian sturgeon fingerling was earlier found to be significantly increased by dietary PHB, but only when supplemented at a high level of 50 g kg^{-1}. A variety of lipases and colipases are involved in the general digestive process for

lipids as they play an important role in the extracellular lipid hydrolysis in the intestine, stomach, and caecal lumen (Trocher, 2003). Hence, it can be that the higher lipase activity resulting from PHB supplementation (Chapter 2) indicates a high activity of lipase in the digestion of PHB as a lipid (Chapter 2). Consequently, we hypothesize that the growth promoting effect of PHB is due to an improved nutrient utilization –particularly in lipids digestion and absorption– that increased lipid deposition in adipose tissue (i.e. triglycerides). It is reported that when the triglycerides are mobilized from the fat tissue, their hydrolysis by the lipase results in the release of free FAs that can be utilized either directly through β-oxidation and serve as fuel mainly for the muscle and also the heart, or indirectly after initial conversion to ketone bodies (Leblanc and Ballantyne, 2000; Phillips and Hird, 1977). Ketone bodies, primarily the β-hydroxybutyrate (β-HB) and its dehydrogenated counterpart acetoacetate (AcAc), can be produced in the liver as transportable units of fat during conditions of high circulation of free fatty acids (Phillips and Hird, 1977). The ketone bodies are transported by the blood to the extrahepatic tissues where they are oxidized via the tricarboxylic acid (TCA) cycle (Ballantyne, 2014; Dedkova and Blatter, 2014) to provide the energy required by the tissues, including the heart (Zammit and Newsholme, 1979), brain and skeletal muscle (Beis et al., 1980). In this study, it can also be that the higher lipase activity in the PHB treatment groups indicates an increase in the hydrolysis of the deposited fat (Chapter 2), which would have provided energy that might have contributed in the higher fish growth (Chapter 3), either through β-oxidation or ketone bodies production. PHB is a fatty acid polymer and can thus be considered to be a typical source of energy (Azain, 2004). In accordance to this, De Schryver et al. (2010) reported that the caloric value of the PHB was slightly higher (22.4 kJ g^{-1}) than that of the basic feed (19.6 kJ g^{-1}). Because fish feed to meet their energy requirements, diets with excessive non-protein energy may result in decreased feed intake and reduced weight gain (Ogunji et al., 2008). Other studies suggested that excess energy relative to protein content in the diet may result in high lipid deposition, which does not equate to faster growth and is an inefficient use of

nutrients (Hixson, 2014). Hanley (1991) indicated that tilapia were able to store significant quantities of lipid in their carcass and viscera, but could not utilize this energy source to improve growth. Overall, the fish lipid content obtained in this study (±6 % WW) is much higher than often reported for tilapia juveniles (2.5-4.5 % WW) (Chou and Shiau, 1996). In this study, even though the protein to energy (P/E) ratio of the experimental diets or the fish was not analysed, PHB supplementation by replacing cellulose in the basal diet might have resulted in excessive energy, reducing the dietary P/E ratio when compared to the basal / control diet, which may have been the reason for the slightly lower ($P > 0.05$) growth performance in the fish group fed with high PHB supplementation (25 g kg^{-1} diet and 50 g kg^{-1} diet) compared to the control group in the first study (Chapter 2). Significantly higher body lipid in fish fed PHB diets may support this speculation. Similar speculation might also be applied in the second study (Chapter 3), where similar growth was observed for treatment group with high PHB supplementation (25 g kg^{-1} diet and 50 g kg^{-1} diet) compared to the low PHB supplementation (1 g kg^{-1} diet to 5 g kg^{-1} diet). As for this speculation, it is important to account for this nutritional parameter in future PHB studies, especially for the interpretation of the effect of dietary PHB on fish growth and fish body composition. Further investigation is needed to evaluate how the PHB contributes to specific nutritional needs (e.g., optimal digestible protein to digestible energy ratios) of tilapia at different life stages. Further studies also need to focus on the fish lipid metabolism with PHB supplementation, for example using the compound-specific isotope analysis (SCIA). Lipids (FAs and sterols) are ideal targets for compound-specific stable carbon isotope analysis since they are abundant in higher organisms (Chamberlain et al., 2004). In such studies using tilapia fish, it is then important to keep in mind that FA $\delta^{13}C$ values are not necessarily reflect those of the dietary FAs, and that one should be aware of the fish bioconversion of FAs activity when interpreting the information on fish FAs profile.

Effects of dietary PHB on the fish gut community-level physiological profiling

As hypothesized earlier, the growth promoting effect of PHB seemed to be associated with an improved nutrient utilization, particularly in the lipid metabolism (Chapter 2). It is suggested that the regulation of intestinal dietary lipid absorption is critical to maintain the host's growth and energy balance (Musso et al., 2011). The intestinal microbiota is suggested to play a role in fish nutrition, including differentiation of the fish intestinal epithelium and to contribute by enzymes or nutrients, which improve feed digestion and nutrient uptake (Denev et al., 2009; Dibner and Buttin, 2002; Ewing and Cole, 1994). Although the microbiota can influence dietary nutrient harvest, the diet can also impact gut MC composition and functionality (Bolnick et al., 2014). De Schryver et al. (2010) reported that the dosage of PHB in the diet of juvenile European sea bass induced a steering effect on the gut MC. The alteration of gut MC by dietary PHB was also observed in sturgeon fingerlings (Najdegerami et al., 2012). Using a gnotobiotic zebrafish model, Semova et al. (2012) hypothesized that diet-induced alterations in gut microbiota composition might influence lipid absorption by several possible mechanisms: (1) by increasing the bioavailability of FAs through modification of the production or composition of bile salts (Swann et al., 2011), (2) by promoting FA availability for potential absorption by direct contribution in lumenal lipolytic activity (Ringø et al., 1995), (3) by enhancing FA absorption indirectly through evoking physiologic responses in the intestinal epithelial cells, and/or (4) by reducing rates of FA oxidation in intestinal epithelial cells permitting increased storage of FAs. In this study, it was initially hypothesized that the increased whole-body lipid content of fish fed with PHB (Chapter 2) might be due to a change in GI microbiota functionality resulting from PHB supplementation, either in terms of intestinal absorption or metabolism of exogenous dietary lipids or in terms of hepatic production or metabolism of endogenous lipids. In order to test the hypothesis, the study described in Chapter 3 aimed at detecting an effect of PHB on the gut microbiota physiological profile. The results could, however, not show any

alteration in the gut MC physiological (functional) profile by the dietary PHB based on a variety of 31 carbon substrates. Hence, it can be suggested that the growth promoting effect of PHB is not necessarily related to changes in the functional gut MC profile or its feed digestion pattern. It should be noted, however, that the observation is based only on the degradation of the carbon sources by a part of the gut MC, namely the aerobic and facultative anaerobic bacteria. The oxidative metabolism of the gut microbiota is distributed between anaerobic and aerobic respiration, with the intermediate case of the facultative anaerobic respiration (Mouchet et al., 2012). Since tilapias are herbivorous / omnivorous and possess a relatively long and coiled gastrointestinal tract (Smith et al., 2000) this would favour the colonization of anaerobic bacteria (Ferguson et al., 2010). This suggests that further study would be needed to evaluate the effect of PHB on the community-level physiological profiling by focusing on the gut anaerobic microbiota (i.e. by using the Biolog AN MicroPlate™ method).

Diet-induced alteration in gut MC composition can possibly occur unless the communities are resistant (composition remain unaltered), resilient (original composition are restored) or functionally redundant (original functions are retained) (Berga et al., 2012). Functional redundancy implies that the new community that is established may be different in composition but performs the same processes as the community prior to modification (Wohl et al., 2004). Allison and Martiny (2008) suggested that a community can only be completely altered if none of the abovementioned characteristics are present. As for this study, further investigation is needed to find out whether the unaltered physiological profile of the gut MC upon dietary PHB supplementation (Chapter 3) was due to the gut MC that was resistant, resilient or functionally redundant. A plausible explanation for a high functional redundancy is given by the multifunctionality of communities (Hector and Bagchi, 2007). This concept posits that low to moderate levels of species diversity are required to support one function, but higher levels are necessary to support all ecosystem functions. Thus, maintaining species sharing a similar function in a given ecosystem but with different environmental

requirements is one of the main mechanisms supporting assemblage stability and resilience by providing substitutes to species lost in fluctuating environments and thus acting as insurance against loss of species diversity (Yachi and Loreau, 1999). This apparent redundancy may be weaker when considering more functions. For example, this study is focusing on the carbon cycle, but the fish gut MC is involved in the nitrogen and sulphur cycle as well. We hypothesized that the fish gut MC may be less functionally redundant than has been revealed by our limited set of carbon sources, when considering (i) a higher number of carbon sources (e.g. Biolog GN-/GP-MicroPlate™ with a set of 95 carbon sources), or (ii) several nutrient cycles. Overall, the results of this study showed no clear effect of dietary PHB towards the fish gut MC physiological / functional profiling. Consequently, the potential impact of dietary PHB on the physiological profile of the gut MC remains unclear. Advance studies will be required to identify the functional relationship between dietary PHB and the gut microbiota of Nile tilapia.

Biodegradation of PHB and its compartmental distribution in fish body

Overall, the information gathered in Chapter 2 and 3 suggests that dietary PHB increased the growth of Nile tilapia, hypothetically by the improvement of nutrient utilization –particularly in the lipid digestion, absorption and metabolism– which is not necessarily associated with the gut MC physiological / functional profile. For a complete understanding of the PHB performance in animal a comprehensive evaluation of the PHB degradation process is necessary. It is generally accepted that biodegradation of PHB both in living systems and in the environment occurs via enzymatic and non-enzymatic processes that take place simultaneously under natural conditions (Bonartsev et al., 2011; Freier et al., 2002; Marois et al., 1999). The degradation rate of PHB is influenced by the characteristics of the polymer, such as chemical composition, crystallinity, morphology and molecular weight (Abe and Doi, 2002).

The examination of hydrolytic degradation of natural PHB *in vitro* is a very important step for understanding PHB biodegradation. The hydrolytic degradation of PHB is usually examined under experimental conditions simulating internal body fluid, in buffered solutions with pH = 7.4 at 37 °C, although higher temperature (55 °C, 70 °C, and more) and other pH values (from 2 to 11) have also been selected (Iordanskii et al., 2014). Several studies suggested that the non-enzymatic hydrolysis of PHB proceeds via random bulk hydrolysis of ester bonds in the polymer chain (Bonartsev et al., 2007; Boskhomdzhiev et al., 2010; Koyama and Doi, 1995). The examination of enzymatic degradation of PHB *in vitro* is the following important step for understanding of PHB performance in animal. Most studies observed degradation of PHB by depolymerases of its own bacterial producers (Bonartsev et al., 2007; Koyama and Doi, 1995). Several studies suggested that the enzymatic degradation of PHB *in vitro* occurs in the presence of various lipases that act as non-specific esterases (Hoshino and Isono, 2002; Qu et al., 2006; Shishatskaya et al., 2005). At the next step it is necessary to observe enzymatic degradation of PHB under the conditions that modelled the animal tissues and body fluids containing nonspecific esterases. Miller and Williams (1987) showed degradation of PHB in the serum and blood, with progressive mass loss of PHB sutures of 16 % and 25 %, respectively, after 180 days of incubation. Other study showed PHB degradation in crude extracts from animal tissues, including liver, kidney, heart, brain and muscle, from 2 to 18 % mass loss of PHB microspheres after 17 h incubation at pH 7.5 and 9.5 (Shishatskaya et al., 2005). Various studies suggested that lipase can penetrate into pores of PHB film but the enzymatic degradation proceeds mainly on the surface of the coarse polymer film achievable for lipase. In addition to studies on PHB hydrolysis and enzymatic PHB degradation *in vitro* by lipases, *in vivo* biodegradation of PHB implant (in the form of plates, films, fibers, monofilament threads, and micro- and nanospheres) has been studied in various laboratory animals, including rats, mice, rabbits, guinea pigs, cats, calves, and sheep (Borkenhagen et al., 1998; Freier et al., 2002; Kunze et al., 2006; Kuppan et al., 2011; Malm et al., 1992; Qu et al., 2006). Pişkin (1995)

reported that the *in vivo* biodegradation of PHB is faster than *in vitro* hydrolysis at body temperature, indicating that enzymes existing *in vivo* catalyze the degradation. Very clear data that clarify the tissue response that contributes to biodegradation of PHB patch after implantation into the gastrointestinal tract of rat was reported by Löbler et al. (2002), where specific genes, namely the vitamin D binding protein mRNA and C-reactive protein mRNA were found to be induced after 7 and 14 days of contact between PHB and rat stomach tissues. Similarly, a specific tissue response induced by an implantation of PHB fiber was reported by Shishatskaya et al. (2005), showing the presence of two types of degradation enzymes, namely liver serine esterases and kidney esterases, in rat stomach tissues. As for tilapia fish, lipase activity has been found in extracts of the pancreas, pyloric ceca and anterior intestine (Ballantyne, 2014; Sargent et al., 2002; Tengjaroenkul et al., 2000). In this study, we hypothesized that the high lipase activity in fish fed with PHB (Chapter 2) demonstrates its high activity in PHB biodegradation.

To actually show whether dietary PHB is digested (degraded), absorbed and metabolized in Nile tilapia body after ingestion, the study described in Chapter 4 aimed at detecting carbon isotope incorporation from the labelled PHB diet in different tissues. As expected, carbon isotope originated from labelled PHB diet was compartmentalized in different tissues at different rates, suggesting that PHB is being digested, absorbed and metabolized in different tissues. As the isotopic incorporation among tissues was found to be fastest in the intestine, followed by the isotopic incorporation in the kidney, liver, and spleen, it can be suggested that these tissues act as the major metabolically active tissues ('source tissues') with clear functional responses to isotopic incorporation from the PHB, while the lower isotopic incorporation in the heart indicates its response as an 'assimilator tissue'. The lowest isotopic incorporation levels were observed in the blood, brain and muscle. The low isotopic incorporation in the blood indicates its function as a 'carrier tissue', which is characterized by a constant uptake and release of C from PHB; while for the brain and muscle the low isotopic incorporation indicates their response as 'sink tissues'.

In this study, we related the observed compartmental distribution of C originating from PHB with the nutrient flow suggested by Halver and Hardy (2002). We further hypothesize the possible metabolic route of PHB in the fish body: that once it is ingested, PHB is digested and absorbed by the lipase in the intestine, resulting in free fatty acids that are transported to the blood for which there are two possible routes; the lymphatic vessels of the intestine and the veins of the portal system. The long-chain fatty acids are transported by the lymphatic system (kidney, spleen) and enter the systemic circulation directly (Sheridan and Friedlander, 1985; Zapata et al., 2006), while short-chain fatty acids are transported to the liver via the hepatic portal vein where they are esterified into triglycerides, converted into cholesterol or phospholipids, or oxidized into ketone bodies, before entering the systemic circulation (Halver and Hardy, 2002). We hypothesize that the produced ketone bodies (particularly β-HB) are circulated by the blood (Hemre et al., 1991; Zammit and Newsholme, 1979) and further utilized mostly by the heart (Hemre et al., 1991; Leblanc and Ballantyne, 2000), and also by the brain and muscle as their energy sources (Beis et al., 1980; Magnoni et al., 2001; Soengas and Aldegunde, 2002).

Possible mode of action of PHB as a growth promoter

It was first established that PHB improves growth of Nile tilapia fry (Chapter 2) and fingerlings (Chapter 3). This coincided with an increase in lipase activity as well as with an increase in whole-body lipid deposition (Chapter 2). This suggests that PHB is either considered and degraded in the body as a fat or that it influences the fat metabolism of the fish. The results from the ^{13}C-PHB incorporation experiment (Chapter 4) suggest the important role of intestine in the digestion and absorption of PHB as a fat, as well as the important role of the liver in the PHB metabolism as a lipid. It was hypothesized earlier that the higher total whole-body lipid content in the PHB treatment groups may be due to larger amount of visceral fat, which is deposited in the adipose tissue in the form of fat (triglycerides). These triglycerides are an enormous reservoir of metabolic fuel. Consequently, we

hypothesize that the growth promoting effect of PHB can be due to the high energy delivered by the PHB as a lipid.

PHB supplementation may potentially result in the release of SCFA and so the growth promoting effect of PHB might be explained by the benefits associated with the availability of SCFA in the gastrointestinal tract. In herbivorous mammals, such as ruminants with voluminous large intestines, SCFA production accounts for as much as 80 % of maintenance energy requirements (Engelhardt and Rechkemmer, 1985). The supplementation of sodium butyrate at 30 g kg^{-1} in the diet of calfs also resulted in the improvement of growth and nutrient utilization (Ślusarczyk et al., 2010). Butyrate was reported to be one of the main precursors, next to acetate, of the *de novo* synthesis of lipids in colonic epithelial cells in rats (Zambell et al., 2003). The SCFA butyrate is essential for maintaining the normal metabolism of intestinal mucosa and regulating cellular growth / cell membrane synthesis (Kruh et al., 1994). Thus, we hypothesize that dietary PHB induce SCFA production that stimulate the epithelial cell growth in the intestine and increase the uptake efficiency of nutrients from the feed, and thereby promoting the fish growth. This, however, needs further investigation.

Furthermore, we further hypothesize that the growth promoting effect of PHB could also be due to the production of the intermediate product of PHB biodegradation, ketone body β-hydroxybutyrate, and its use as an energy source by the muscle, as well as by the vital organs, such as the heart and brain (Willmott et al., 2005).

Effectiveness and efficiency of PHB application as a growth promoter

The biodegradation of PHB also depends on the physical properties of the polymer (Boskhomdzhiev et al., 2010; Freier et al., 2002; Vroman and Tighzert, 2009). Intracelullar PHB granules are insoluble and coated with phospholipids and proteins which stabilize it (Wieczorek et al., 1995). There are seven different structural proteins (encoded by 'phasin'; inclusing phasin PhaP1 to PhaP7) that can

be detected at the surface of PHB granules during *in vivo* or *in vitro* studies, with the amount of PhaP1 at the surface of PHB granules by far being the largest in comparison to the amounts of other phasins (Pfeiffer and Jendrossek, 2011; 2012; Pötter et al., 2004). It is reported that the amorphousness of intracellular PHB granules is stabilized by a small amount of water (5-10 %) and these phasins in the membrane lipids. The removal of these impurities induce partial crystallization which toughens the granules and inhibit extensive molecule degradation and granule aggregation (Porter and Yu, 2011). It is reported that these phasins also seemed to have a regulatory influence on the degradation of PHB, although the exact mechanisms remain unknown. The lack of PhaP1 caused a reduced *in vitro* degradation of native PHB granules (nPHB) in *Ralstonia eutropha*, while the lack of all phasins considerably reduced the degradation of nPHB although the degradation was not fully prevented (Eggers and Steinbüchela, 2013; Uchino et al., 2007). These findings suggest that the cPHB might be degraded differently from the amorphous PHB (i.e. less hydrolyzed). In this study, the increase in lipase activity (Chapter 2) indicates *in vivo* cPHB enzymatic biodegradation. The incorporation of the ^{13}C stable isotope originating from labelled PHB diet in different Nile tilapia tissues, i.e. intestine, liver, kidney, spleen, heart, blood, brain, and muscle (Chapter 4), also strongly indicated *in vivo* aPHB biodegradation, which contributed to tissue formation. It is hypothesized that the growth modulation effect of PHB observed in Nile tilapia fry and fingerling (Chapter 2 and 3) could have been even higher by using aPHB instead of cPHB due to the hypothetically higher degradation / hydrolysis of aPHB compared to cPHB. However, further investigations are needed to confirm the hypothesis.

In term of cost effectiveness, the use of bacterial cells that contain aPHB can be considered as an effective alternative for cPHB particles (Halet et al., 2007). It is reported that the extraction of PHB from the cells alone costs about 30 % of the total production cost of the pure / crystalline PHB (Mudliar et al., 2008). Consequently, the absence for the need of PHB extraction in the application of amorphous PHB will considerably decrease the total production costs of PHB. It was estimated that

the price of PHB still contained within bacterial cells will be about 40 % lower than that of pure extracted crystalline PHB (De Schryver, 2010). Overall, more research is needed for an understanding of PHB degradation and metabolism, enzymatically or physically, by the fish (host) and/or its associated microbiota, in order to effectively and efficiently apply PHB as a growth promoter in aquaculture production.

Evaluation of PHB as a health promoter in aquaculture

Development of gnotobiotic tilapia larvae system

In this study, in addition to its growth promoting effect (Chapter 2 and 3), PHB was also studied for its health promoting effect (Chapter 5). In order to design efficient PHB treatment strategies for addressing health problems in aquaculture, one would need an increased understanding of the interactions between dietary PHB, host, and gut microbiota. To clarify these different contributing factors / interactions that make up the mechanism of action of PHB, it is important to exclude any arbitrary influences during the investigations. The use of gnotobiotic animal models in which the composition of the bacteria is exactly known allows an increased control over the variables and an enhanced reproducibility of results. Gnotobiotic animal models have been applied in different categorical studies: (1) nutrient requirements, (2) metabolic functions, and (3) complex host–microbe interactions (Dierckens et al., 2009; Forberg et al., 2011; Kau et al., 2011; Marques et al., 2006; Rawls et al., 2004; Semova et al., 2012; Yossa et al., 2013). Focussing on tilapia, as this is the test organism used in this work, the main benefit associated with the use of the gnotobiotic tilapia larvae as compared to other gnotobiotic fish larvae, i.e. gnotobiotic cod or gnotobiotic seabass larvae, is that these larvae can take up either live feed (i.e. *Artemia* nauplii) or formulated microdiet at first feeding. This is important as the use of formulated diet in gnotobiotic system allows us to control not only the microbiological, but also the dietary variable which might have potential effects on host metabolism. In this study (Chapter 5), gnotobiotic tilapia larvae system was obtained by a double egg surface disinfection

procedure using hydrogen peroxide (2000 mg L^{-1} for 10 min) and sodium hypochlorite solution (75 mg L^{-1} active chlorine for 2 min), with the use of a cocktail of antibiotic (ampicillin, rifampicin, kanamycin, trimethoprim and gentamycin each at 10 mg L^{-1}) and antifungal agents (Amphotericin-B and Fluorescent Brightener-28 at 0.5 mg L^{-1} and 25 mg L^{-1}, respectively) to maintain gnotobiotic condition.

Application of gnotobiotic tilapia system to investigate the protective effect of PHB

Similar to the study with giant freshwater prawn larvae (Thai et al., 2014), PHB also acted as a protecting agent for gnotobiotic Nile tilapia larvae. As illustrated in Chapter 5, dietary PHB induced an increase in larval tilapia survival with about 20 % in a gnotobiotic challenge test with pathogen *E. ictaluri* gly09R. However, the direct effect of PHB on fish morphological / physiological responses is non-exclusive as the PHB might also have directly affected the pathogen. As for this, it will be interesting to use the gnotobiotic tilapia system that has been developed in this study to clarify the different possible mechanisms of action of PHB in the improvement of the fish disease resistance and overall immune status. For this purpose, the gnotobiotic tilapia larvae system can be used (i) to study the interactions between dietary PHB and host, (ii) to study the interaction between dietary PHB and gut microbiota, (iii) to study the modulation of gut microbiota and host metabolic interactions, and (iv) to study to which extent interactions between gut microbiota and dietary PHB can affect host physiology and health.

In Figure 6.1 an overview is provided on the different elements that should make up the study on the mechanistic action of PHB using gnotobiotic tilapia larvae as test organism. The antimicrobial effect of PHB (and its degradation products; β-hydroxybutyrate) towards the survival and infection of *Edwardsiella ictaluri* (*Edwardsiella* spp.; tilapia intracellular pathogens) need to be investigated. A study using green fluorescent protein (GFP) showed that both avirulent and virulent *Edwardsiella* sp. are able to adhere to, invade, and replicate in the fish epithelial

papilloma (EPC) cell line using host microfilaments and protein tyrosine kinase (Ling et al., 2000). Type III secretion system (T3SS) and type VI secretion system (T6SS) play important roles in adherence, penetration, survival, and replication of *E. tarda* in epithelial cells and phagocytes. The T6SS of *E. tarda* comprises 16 genes, and 13 of the encoded proteins are involved in the secretion of EvpP (*E. tarda* virulence protein) (Zheng and Leung, 2007). T3SS is a multi-protein complex that is essential for host and pathogen interaction. In *E. tarda,* T3SS proteins include the *E. tarda* secretion system apparatus (EsaB and EsaN), effectors (EseB, EseC and EseD), chaperones (EscA, EscB and EscC), and regulators (EsrA, EsrB and EsrC) (Wang et al., 2010; Zheng et al., 2007). Proteomic studies have revealed that EseB, EseC and EseD are the major extracellular protein, and mutations of these genes in *E. tarda* reduce virulence compared to parental *E. tarda* (Tan et al., 2002). These pathogenic genes expression may thus be of interest in the future PHB studies on tilapia pathogen *Edwardsiella* sp.

At the level of tilapia, the easiest factors to score are survival and growth. Future study, however, also needs to focus on the fish immune responses that may provide protection against this intracellular bacterial infection. Several immune-related genes, such as Notch2 and nfatc3b, have shown crucial role in immune regulating function in tilapia (Xiao et al., 2015) and thus may be of interest for future studies.

It has been accepted that the intestinal epithelium acts as the major physical barrier to pathogen infection, and it is the location of competitive exclusion by the autochtonous microbiota, and production of antimicrobials (Cain and Swan, 2011; Esteban, 2012). This is of importance as the intestinal tract is considered as the main portal of entry to many enteric pathogens, including *E. ictaluri* (Li et al., 2012; Soto et al., 2013), which was used in the challenge tests on gnotobiotic Nile tilapia larvae (Chapter 5). Using gnotobiotic tilapia, further study can focus on the effects of PHB and its degradation products on the development of the gut by histological and stereological analysis (Najdegerami et al., 2013; Rekecki et al., 2009). This may show

whether or not the fish morphology is beneficially influenced, which may also provide protection against intracellular bacterial infection. The gut volume to body volume ratio, the intestinal epithelium height, the specific surface area of the villi , the villi number,... are all factors that can be investigated. The use of gnotobiotic tilapia larvae is ideal for this future study, as there will be no interference by microbiota on the proliferation of gut epithelial cells, as often described in the conventional system (Denev et al., 2009).

The information about the exact mechanisms of action of PHB will provide a better understanding of how to extract the full potential of PHB applications for health promotion in aquaculture production. However, one must consider that the functioning of metabolic processes in axenic / gnotobiotic animals is likely much different from their conventional counterparts. Thus, the gathered information obtained from gnotobiotic studies on PHB needs to be validated for conventional / non-bacteria free animals.

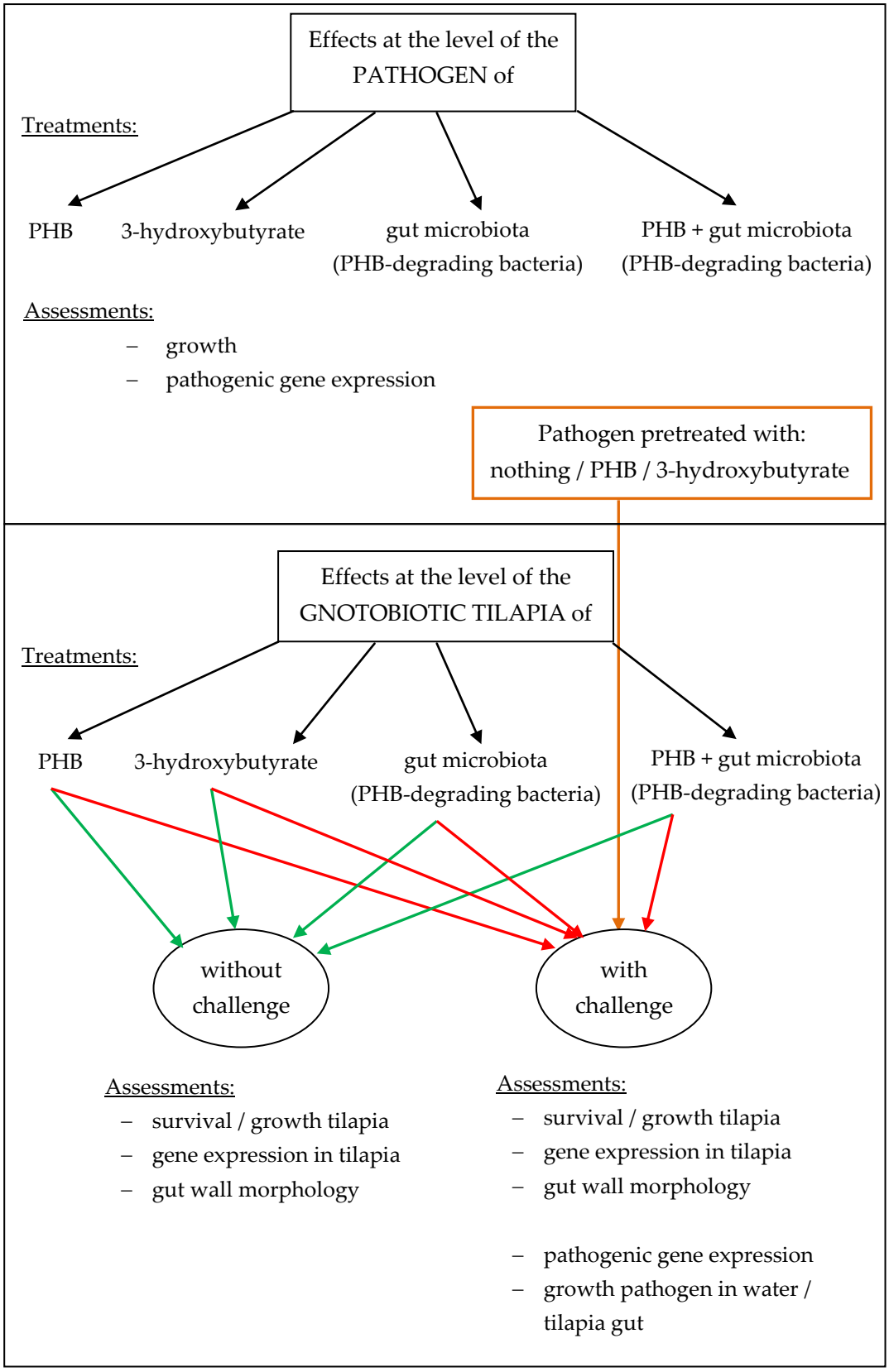


Figure 6.1. Proposed research scheme on the mode of action of PHB using the gnotobiotic tilapia larvae system (as suggested in De Schryver, 2010).

Possible mode of actions of PHB as health promoter

The effect of PHB at the host level is reported by Suguna et al. (2014), whereas PHB increased resistance of Java tilapia against *Aeromonas hydrophila* infections by stimulating both specific immune response (analysed in terms of antibody response to sheep red blood cells) and nonspecific immune response (analysed in terms of serum lysozyme activity, total peroxidases activity and antiprotease activity) when supplemented at a higher dose of 5 g kg^{-1}. Futhermore, PHB may have a direct effect on the host, for example in the alteration of morphological and physiological responses in the intestinal epithelium, which functions affect the immune system.

PHB can also work at the level of pathogen. As suggested by De Schryver et al. (2010), it is hypothesized that PHB degradation in the intestine results in high concentrations of short chain fatty acids like β-hydroxybutyric acid and/or its oligomers (Chapter 2). β-hydroxybutyric acid is known to exhibit some antimicrobial, insecticidal, and antiviral activities (Tokiwa and Ugwu, 2007). The antimicrobial activity of β-hydroxybutyric acid is thought to be comparable to that of other short-chain fatty acids (SCFA) (Defoirdt et al., 2007a). Another PHB effect at the level of pathogen is reported by Kiran et al. (2014), where PHB (originated from *Brevibacterium* sp.) significantly reduced biofilm formation of *Vibrio* sp. on PHB-coated glass and polystyrene plates, suggesting that PHB may potentially reduce the colonization capacity of *Vibrio* sp. in the intestine of shrimp culture and thus reduce the biomass establishment – a prerequisite for virulence expression – of the pathogen. It was suggested that the use of PHB to prevent pathogen biofilm formation instead of inhibit its growth could serve as an alternative to conventional biocontrol procedures using antimicrobial compounds (Kiran et al., 2014).

It was hypothesized earlier that PHB degradation might result in the production of ketone body β-HB (Jendrossek & Handrick, 2002) (Chapter 2 and 4). Defoirdt et al. (2009) suggested that β-HB has been reasoned to protect aquaculture animals from bacterial infections in two ways: (1) by decreasing the growth and/or the virulence of the pathogens (effect at the pathogen level), and (2) by providing energy to the

host intestinal mucosa, thereby increasing intestinal health and resistance to infections (effect at the host level). A study by Dong et al. (2012) using rats suggested that the increased levels of ketone body 3-HB in plasma of rats with *Klebsiella pneumoniae* infection showed a strong increase in β-oxidation and a drop in glucose concentration, which could mirror the high demand of the body for energy in response to bacterial infection. It is known that the oxidation of fatty acid produces more energy per molecule than glycolysis (Trocher, 2003), therefore ATP generated from fatty acid oxidation is an important energy source required by the major organs (e.g. liver, heart, spleen and kidney) to function during severe sepsis. 3-hydroxybutyrate is also known as keton body used as energy source in several tissues in fish (Willmott et al., 2005). The energy supply from the ketone body 3-HB is suggested as the important fuel for development and growth of *Artemia* culture (Weltzien et al., 2000), which could also be responsible for the increase in growth of giant freshwater prawn larvae fed with PHB-supplemented *Artemia* (Nhan et al., 2010; Thai et al., 2014). It is thus hypothesized that a shared mechanism of PHB and/or its monomer 3-HB could have been responsible for the health as well as growth promotion in tilapia culture as observed in this study, by (1) providing the antimicrobial activity, and (2) providing energy to support body function during infection (sub-optimal) as well as to enhance growth during normal (optimal) conditions.

General conclusions

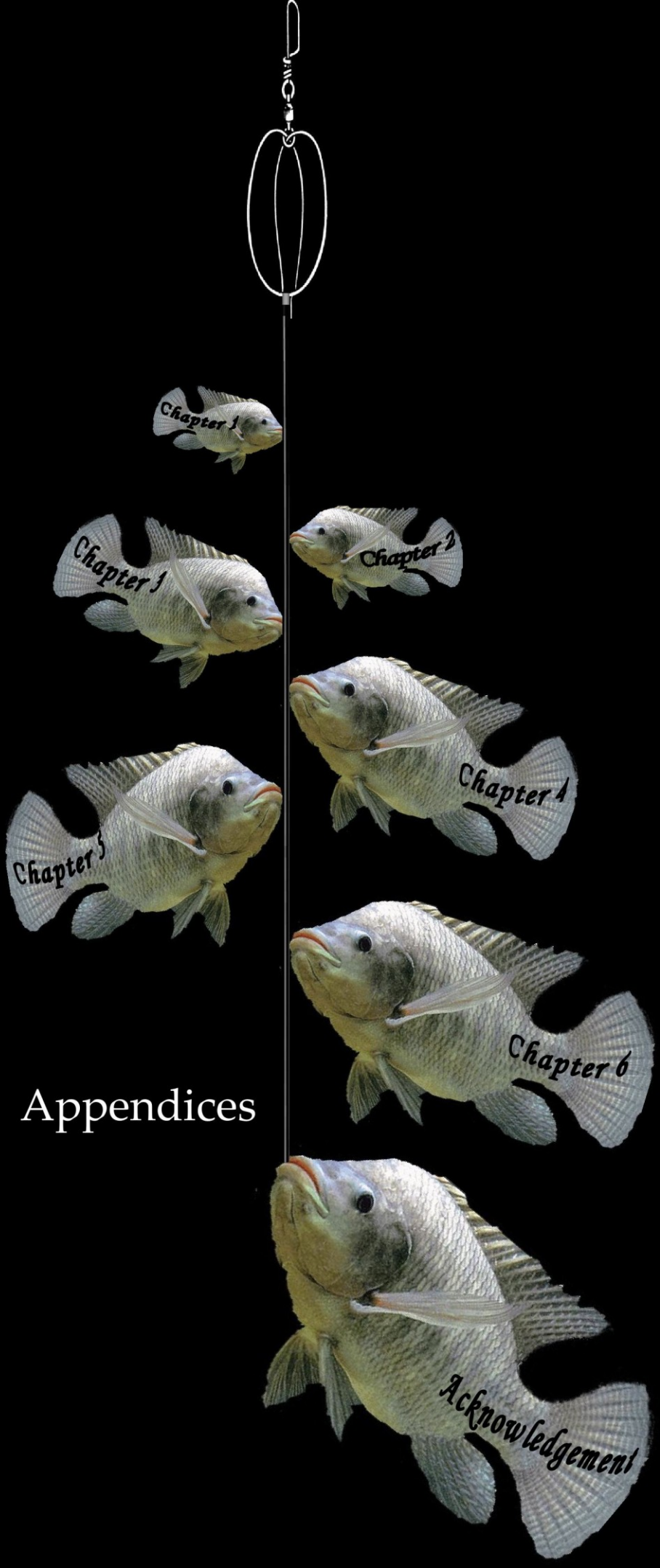
Considerable efforts have been made to render aquaculture more sustainable, due to the fast expansion of the aquaculture production that has exerted high pressure on the environment. On one hand, this implies the search for (micro-)biologically based methods to limit the use of antibiotics as growth promoters for food animals and/or to control pathogenic infections. As for this, the bacterial storage compound poly-β-hydroxybutyrate has been proposed as an alternative to antibiotics and studied in several main aquaculture species. This research contributed to the exploration of PHB as it showed that:

- PHB can have a growth promoting effect when supplemented to the diet of tilapia fingerlings
- PHB can potentially improve the nutrient utilization, particularly in lipid metabolism
- PHB is metabolized, compartmentally distributed and incorporated in different organs / physiological systems
- PHB can be used in a gnotobiotic system to confirm its protective effect against pathogenic infection

The exact mechanisms of action of PHB, however, require further thorough exploration in order to optimally apply PHB at full-scale. Further research is required to explore and understand the full potential of PHB as a growth and/or health promoting agent in wide aquaculture practices. This should focus on:

- determining the optimal developmental stage at which PHB should/can be applied for each targeted animal / species
- unravelling the mechanistic action of PHB on fish nutrient utilization, particularly the lipid metabolism, e.g. by applying the whole-body fatty acid balance method (WBFABM), an *in vivo* method for assessing fatty acid metabolism, including rates of liponeogenesis and *de novo* fatty acid production, β-oxidation and also bioconversion (elongation and desaturation) of fatty acids

- detecting the effect of PHB on the physiological profile of fish gut microbiota, including the more diverse microaerophilics, e.g. by using the Biolog GN-/GP-MicroPlate™ method (with 95 C sources; instead of 31 C sources as in Biolog EcoPlate™), as well as the anaerobics, e.g. by using the Biolog AN MicroPlate™ method.
- understanding the biodegradation processes and the metabolic fate of dietary PHB in the fish body, e.g. a complete mass balance experiment by integrating the compound-specific isotope analysis (CSIA) with the advanced metabolomics approach
- elucidating the mode of action of PHB in its protective effect against pathogenic infection, e.g. by optimizing and making use of the gnotobiotic tilapia system to study (i) the interactions between dietary PHB and host, (ii) the interaction between dietary PHB and gut microbiota, (iii) the interaction between modulation of gut microbiota and host physiology, and (iv) to which extent interactions between gut microbiota and dietary PHB can affect host physiology and health
- rendering PHB more cost effective through the integration into existing microbial control strategies, i.e. by using the bio-flocs technology / BFT for an economical PHB accumulation, or by applying existing PHB-degrading probiotics treatments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Acknowledgement

Appendices

APPENDIX I:

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APPENDIX II:

Summary / Samenvatting

Summary

The present study demonstrated the benefits of the application of PHB in Nile tilapia production. It was confirmed that PHB can significantly improve the growth performance of Nile tilapia fingerling (Chapter 3). PHB resulted in increasing body weight gain in Nile tilapia juvenile, but the difference was not significant when compared to the control group (Chapter 2). In the attempt to explain the growth promoting effect of PHB, the effect of PHB on fish digestion and metabolism, and also gut physiological MC profile have been elucidated (Chapter 2 and Chapter 3). This study showed that PHB improved the nutrient utilization by increasing the lipase activity of Nile tilapia juvenile, which indicates an increase in lipid digestion that could have been lead to the increase in the total lipids content in Nile tilapia juvenile. PHB also resulted in increasing total lipid content in Nile tilapia fingerling, but the difference was not significant when compared to the control group. Furthermore, PHB also seemed to alter the content of several FAs, particularly the total saturated, total monounsaturated and total (n-6) FAs of both Nile tilapia juvenile and fingerling, although the composition of these FAs in the total lipid remain unaltered.

Based on these findings, we hypothesized that the growth promoting effect of PHB is due to an improved of nutrient utilization, particularly with the improvement of lipids digestion, absorption, and metabolism. Although various studies acknowledged the dietary modulation of fish gut microbiota, which seemed to alter the host metabolism and digestion, in this current study we could not detect any effect of PHB on either the resident or the transient gut MC functional physiological profile, suggesting that the growth promoting effect of PHB was not necessarily related to changes in the functional gut MC profile or its feed digestion pattern (Chapter 3). With this knowledge in mind, we hypothesized that the growth promoting effect of PHB is due to physiological response of the fish, instead of its intestinal microbiota.

Based on the carbon isotope tracer study, we demonstrated that PHB is digested and compartmentalized in different tissues at different incorporation rates (Chapter 4). The higher and faster isotope incorporation in the intestine and liver suggest that PHB are highly digested, absorbed and metabolized in these major metabolically active organs. Based on the general knowledge on the main functions of intestine and liver in fish nutrient metabolism, this again suggests the role of PHB in the improvement of lipids digestion, absorption and metabolism, as indicated in Chapter 2.

The present research also demonstrated that the consumption of PHB by gnotobiotic Nile tilapia larvae resulted in higher resistance against infectious *Edwardsiella ictaluri* bacterial challenge (Chapter 5). The developed gnotobiotic Nile tilapia model can be used to further evaluate whether the PHB has a direct effect on the pathogen and/or the host. It is important to note that the magnitude of the expected beneficial effects of PHB on health promotion of commercial Nile tilapia culture (open system) should be further evaluated by considering different operational parameters applied in the aquaculture system.

In conclusion, the overall results of the present research suggest that the application of PHB positively contributed to the growth and health promotion in Nile tilapia culture. To this end, it can only be hypothesized that dietary PHB directly affect the physiological response of the fish in term of nutrient, e.g. fatty acids, utilization / metabolism; possibly with the release of SCFA. It is also hypothesized that the degradation of PHB resulted in the release of its monomer β-hydroxybutyric acid. It is noticed that more research is needed to elucidate the biodegradation processes and the metabolic fate of dietary PHB in the fish body, and to decipher the complex functional relationships between dietary PHB, gut microbiota, and fish physiology, in order to have a better understanding of how to extract the full potential of PHB applications as growth and health promoter in aquaculture production.

Samenvatting

De huidige studie toont de voordelen aan van de toepassing van PHB bij de kweek van Nijltilapia. Het werd bevestiged dat PHB de groei van Nijltilapia juvenielen significant kan verbeteren (Hoofdstuk 3). PHB resulteerde eveneens in een verhoging van de toename van het lichaamsgewicht van Nijltilapia juvenielen, maar het verschil was niet significant in vergelijking met de controle groep zonder PHB supplementatie (Hoofdstuk 2). In een poging om het groeibevorderende effect van PHB te verklaren, werd het effect van PHB op de vertering en het metabolisme van de vissen, alsook op het intestinaal fysiologisch profiel van de gastrointestinale microbiële gemeenschap opgehelderd (Hoofdstuk 2 en Hoofdstuk 3). Deze studie toonde aan dat het nutriëntgebruik door PHB werd verhoogd door toename van de lipase activiteit in Nijltilapia larven. Dit wees op een toegenomen vetvertering die mogelijks de verklaring was van het toegenomen vetgehalte in de Nijltilapia larven. PHB resulteerde eveneens in een toegenomen vetgehalte in Nijltilapia juvenielen, maar het verschil was niet significant in vergelijking met een controle groep zonder PHB supplementatie. Verder leek PHB het vetzuurprofiel van het volledige lichaam van de vissen te veranderen, vooral met toenemende trends in het gehalte aan verzadigde, mono-onverzadigde en n-6 vetten in zowel Nijltilapia larven als juvenielen. Gebaseerd op deze vindingen werd de hypothese vooropgesteld dat het groeibevorderende effect van PHB het gevolg is van een toegenomen nutriëntgebruik, vooral als gevolg van een verbeterde vetvertering, -absorptie, en –metabolisme.

Hoewel verschillende studies de verandering van de microbiële gemeenschap in de darm van vissen, en de daarbij horende metabolisering en vertering, als gevolg van verandering in dieet erkennen, werd in deze studie geen effect gevonden van PHB op het fysiologisch profiel van zowel de residente als transiënte intestinale microbiële gemeenschap. Dit suggereert dat het groeibevorderende effect van PHB niet noodzakelijk het gevolg is van veranderingen in het functionele profiel van de

intestinale microbiële gemeenschap of het verteringspatroon (Hoofdstuk 3). Met deze kennis in gedachten werd de hypothese vooropgesteld dat het groeibevorderende effect van PHB resulteert uit een fysiologische respons van de vissen in plaats van de intestinale microbiota.

Gebaseerd op de koolstof isotoop traceerstudie hebben we aangetoond dat PHB wordt verteerd en verdeeld over verschillende weefsels aan verschillende incorporatiesnelheden (Hoofstuk 4). De hogere en snellere isotoopincorporatie in de darm en lever suggereert dat PHB sterk wordt verteerd, geabsorbeerd en gemetaboliseerd in deze hoog metabolisch actieve organen. Gebaseerd op algemene kennis over de functies van de darm en lever in het nutriëntmetabolisme van vissen, duidt dit opnieuw de rol van PHB in vetvertering, absorptie en metabolisme zoals aangeduid in Hoofdstuk 2.

Het huidige onderzoek toont eveneens aan dat de consumptie van PHB door gnotobiotische Nijltilapia juvenielen resulteerde in een verhoogde weerstand tegen infecties door *Edwardsiella ictaluri* (Hoofdstuk 5). Het ontwikkelde gnotobiotische Nijltilapia model kan verder gebruikt worden om het directe effect van PHB op de pathogeen en/of de gastheer na te gaan. Het is belangrijk om te benadrukken dat de mate van de voordelige effecten van PHB op de gezondheid bij commerciële Nijltilapia cultuur (open systemen) verder dienen te worden geëvalueerd door de verschillende operationele parameters in aquacultuursystemen in beschouwing te nemen.

Concluderend tonen de algemene resultaten van het huidige onderzoek aan dat de toepassing van PHB op positieve wijze bijdraagt tot de groei en gezondheid in de Nijltilapia kweek. In dit kader kan als hypothese gesteld worden dat PHB een directe invloed heeft op de fysiologische respons van vissen in termen van nutriëntgebruik en – metabolisme (bv. vetzuren); mogelijks met vrijstelling van kortketen vetzuren. Een alternatieve hypothese is dat de degradatie van PHB resulteert in de vrijstelling van zijn monomeer β-hydroxyboterzuur. Er is meer onderzoek nodig om de exacte biodegradatieprocessen en het metabolische lot van PHB gedoseerd via het dieet in het lichaam van vissen op de helderen, en om de

complexe functionele relaties tussen PHB, de darmbacteriën en de fysiologie van de vis uit te klaren. Dit zal bijdragen om het volledige potentieel vervat in PHB als groei- en gezondheidsbevorderend middel in de aquacultuur te verstaan en te gebruiken.

APPENDIX III:

Curriculum Vitae

Curriculum Vitae

MAGDALENA LENNY SITUMORANG

School of Life Sciences and Technology
Bandung Institute of Technology
Bandung, Indonesia
situmorangml@sith.itb.ac.id

EDUCATION

Ghent University, Belgium **2010 – 2015**
Ph.D in Applied Biological Sciences
Ph.D thesis:
"Application of poly-beta-hydroxybutyrate (PHB) in growth and health promotion of Nile tilapia *Oreochromis niloticus* culture**"**

Ghent University, Belgium **2006 – 2008**
Master of Science in Aquaculture
Thesis: "The effect of dietary C/N ratio on the performance of the periphyton-based recirculating aquaculture system (RAS) holding Nile tilapia (*Oreochromis niloticus*)"

Bandung Institute of Technology, Indonesia **2001 – 2006**
Bachelor in Biology

AWARDS

LARVANET - NUTRILARVI training school and travel grant **2011**
LARVANET - LARVITA training school and travel grant **2010**
VLIR-UOS ICP PhD Scholarship award **2010 – 2014**
VLIR-UOS Master Course Scholarship award **2006 - 2008**

PROFESSIONAL EXPERIENCE

Bandung Institute of Technology, Indonesia **Academic Assistant**	**2008 – 2010**
Bandung Institute of Technology, Indonesia **Practicum Assistant**	**2005 – 2006**

PROFESSIONAL TRAINING

Marine Biology Research Group, Ghent University, Belgium. **MARES Expert training "Ecological Applications of Biomarkers in Aquatic Food Web Studies"**	**2013**
Laboratory of Aquaculture & Artemia Reference Center (ARC), Ghent University, Belgium. **PROMICROBE PhD course and Training School "Microbial community management in aquaculture"**	**2012**
University of Las Palmas de Gran Canaria (ULPGC), Las Palmas, Canary Islands, Spain. **NUTRILARVI Fish Larvae Training School "Enrichments and micro diets preparation for emerging marine species in aquaculture"**	**2011**
Centre of Marine Sciences (CCMAR) - Aquaculture Research Group and the National Institute of Biological Resources (INRB, I.P.) / IPIMAR - Aquaculture Research Station, Portugal **LARVITA Fish Larvae Training School**	**2010**

SCIENTIFIC PUBLICATIONS AND PAPERS

Situmorang, M.L., Dierckens, K., Mlingi, F. T., Van Delsen, B., Bossier, P. 2014. Development of a bacterial challenge test for gnotobiotic Nile tilapia *Oreochromis niloticus* larvae. Diseases of Aquatic Organisms 109: 23–34

Situmorang, M.L., Dierckens, K., De Schryver, P., Bossier, P., 2015. Poly-β-hydroxybutyrate supplementation in Nile tilapia *Oreochromis niloticus* juvenile culture. Aquaculture, submitted.

Situmorang, M.L., Mebratu, A.T., Dierckens, K., Bossier, P., De Schryver, P., 2015. Use of community level physiological profiling of the gut microbiota in Nile tilapia *Oreochromis niloticus* fingerlings to investigate the growth promoting effect of poly-β-hydroxybutyrate. Applied Microbiology and Biotechnology, submitted.

Situmorang, M. L., Dierckens, K., Ekasari, J., Bossier, P., De Schryver, P. The compartmental distribution of poly-β-hydroxybutyrate after ingestion by Nile tilapia *Oreochromis niloticus* fingerling as monitored by stable ^{13}C isotope labelling. (*in preparation*)

PARTICIPATIONS TO NATIONAL AND INTERNATIONAL MEETINGS AND WORKSHOPS

Situmorang, M. L., Dierckens, K., Mlingi F. T., Van Delsen, B., Bossier, P. 2014. Development of a bacterial challenge for gnotobiotic Nile tilapia *Oreochromis niloticus* larvae. European Aquaculture Society - Aquaculture Europe 2014. Donostia–San Sebastian, Spain. 14 – 17 October 2014. Participant.

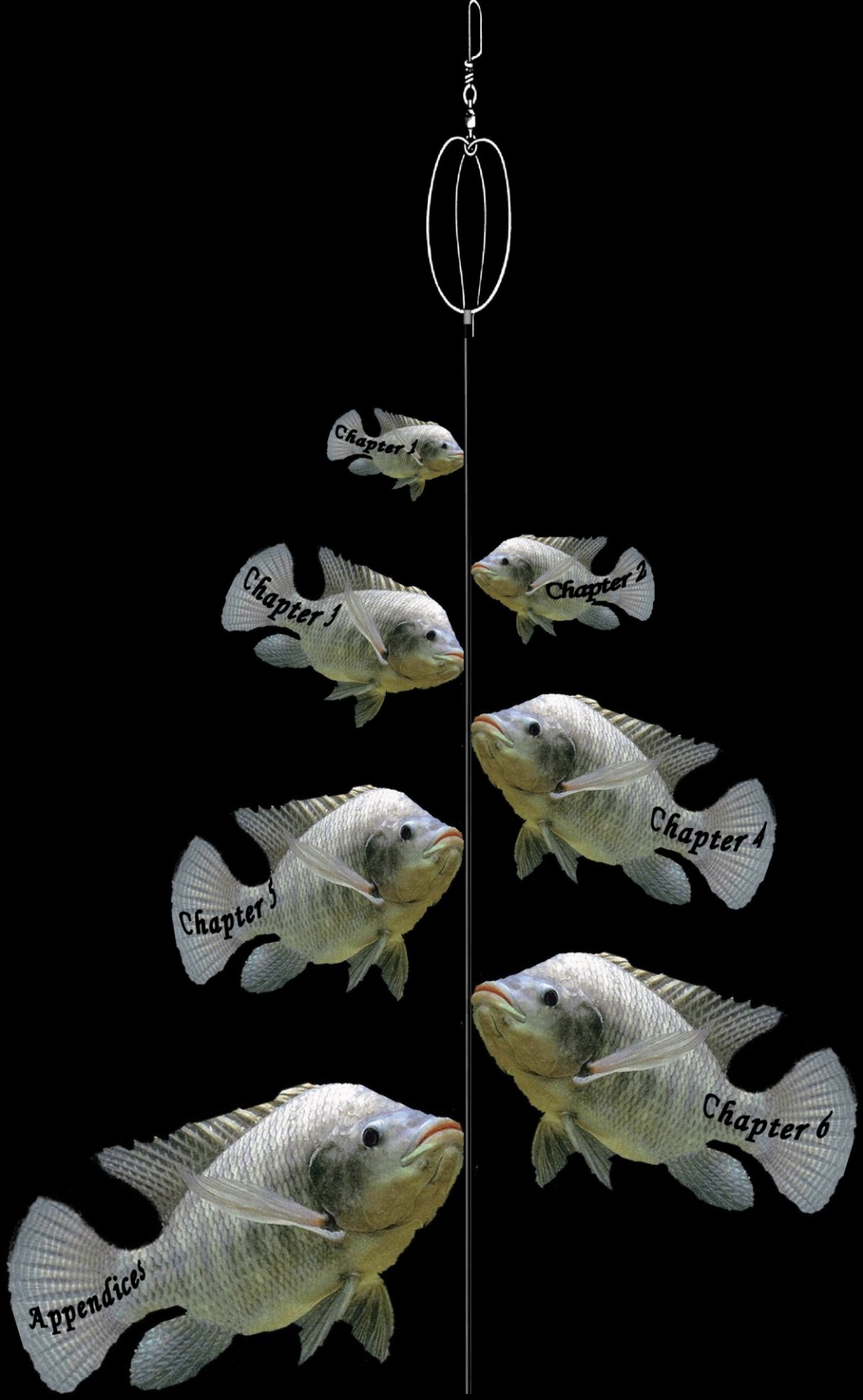
Situmorang, M. L., Dierckens, K., De Schryver, P., Bossier, P. 2013. The effects of dietary poly-beta-hydroxybutyrate (PHB) on growth, feed utilization, survival and digestive enzyme activity of Nile tilapia *Oreochromis niloticus* juvenile. World Aquaculture Society, Asia-Pacific Chapter. Ho Chi Minh City, Vietnam, 10 – 13 December 2013. Oral Presentation.

Situmorang, M. L., Dierckens, K., De Schryver, P., Bossier, P. 2013. The effects of dietary poly-beta-hydroxybutyrate (PHB) on growth, feed utilization, and survival of Nile tilapia *Oreochromis niloticus* juvenile. Larvi 6th Fish & Shellfish Larviculture Symposium, Ghent University, Ghent, Belgium, 2 – 5 September 2013. Poster presentation.

MEMBERSHIPS

World Aquaculture Society

Indonesian Society for Scientific Aquaculture



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Lenny

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